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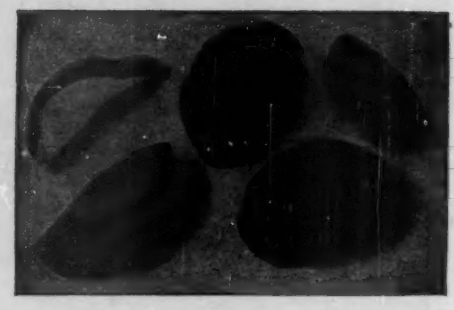

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EFFECT OF CONCENTRATION, EXPOSURE TIME, TEMPERATURE, AND RELATIVE HUMIDITY ON THE TOXICITY OF SULFUR DIOXIDE TO THE SPORES OF BOTRYTIS CINEREA

H. Melvin Couey and M. Uota

U.S. Horticultural Field Station, Fresno, California.
Market Quality Research Division, Agricultural Marketing Service, U.S.D.A.
Respectively, Plant Physiologist and Horticulturist.
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SUMMARY

When spores of *Botrytis cinerea* are exposed to SO_2 gas, the subsequent reduction in spore germination is quantitatively proportional to the SO_2 concentration and the exposure time. The toxicity of SO_2 increases with increasing relative humidity. In an atmosphere of 96% RH, SO_2 is more than 20 times as effective as at 75% RH. The toxicity also increases about 1.5 times for each 10°C rise in temperature between 0° and 30°C.

INTRODUCTION.—SO_2 fumigation to control decay of vinifera table grapes caused by *Botrytis cinerea* Fr. is an established commercial practice in California. The grapes are usually treated immediately after harvest and at intervals during storage (8). The initial SO_2 treatment may be applied in special gassing rooms, precooling rooms, storage rooms, trucks, or railroad cars. When properly done, this treatment kills fungi on or near the surface of the fruit, but does not eradicate disease organisms that have become established in the fruit (7). The initial fumigation is sometimes applied under uncontrolled conditions that contribute to irregular control of decay and to injury of the fruit. Subsequent fumigations of the grapes in storage restrict the spread of infections remaining after the initial treatment.

Although the effect of SO_2 fumigation on subsequent decay of grapes has been studied quite extensively (8), no attempt has been made to determine the effect of SO_2 on the spores and mycelium of any pathogen of grapes under controlled conditions. Therefore, we studied the effect of concentration, exposure time, temperature, and relative humidity on the toxicity of SO_2 gas to the spores of *B. cinerea*.

METHODS.—The culture of *B. cinerea* used in these experiments was isolated from an infected Emperor grape and was maintained on potato-dextrose agar by mass spore transfer. Cultures were grown at 20°C on 7 ml of medium in a 50-ml jar plugged with cotton. Under these conditions, the fungus grew rapidly and sporulated uniformly, producing mature spores by the tenth day after inoculation. Cultures were started at about 10-day intervals to provide a constant supply of vigorous spores. The spores did not vary significantly in germination or in response to SO_2 until the cultures were more than 30 days old. Cultures older than this were not used.

Before fumigation, the spores were dusted lightly on a patch of lens paper or 1.5-mil. polyethylene film about 2 cm square. Lens paper was used for fumigation of wet spores and polyethylene for dry spores. The paper and spores were placed momentarily on moist filter paper and were moistened by capillary action. The paper or polyethylene patches were usually placed on a microscope slide during fumigation. The water held the paper on the slide and the polyethylene was held by a small amount of lanolin or stopcock grease.

The fumigation chamber was 2.7 cm in diameter and 18 cm long, having a volume of about 100 ml. A continuous stream of SO_2 gas entered the chamber at the bottom and flowed upward around the spores. The patches holding the spores could be removed at intervals without interrupting the treatment. Since it was impossible to humidify SO_2 gas mixtures directly, the desired concentrations of SO_2 and water vapor were obtained by mixing streams of SO_2 and humidified air. The air was humidified to 97-100% RH before mixing by passage through a series of 3 water bubblers immersed with the fumigation chambers in a stirred, thermostatically controlled water bath. Stock concentrations of SO_2 (8,500, 5,200, 2,500, and 900 ppm) were prepared by diluting pure SO_2 with N_2. The strength of these mixtures was checked by iodine titration (1). By appropriate dilution, many concentrations of SO_2 at various levels of relative humidity were maintained. Flow rates were controlled with 2-stage pressure regulators, needle valves, and rotameter-type flowmeters. The relative humidity levels and SO_2 concentrations given in the text are based on dilution, but also were checked periodically by measuring the relative humidity with thermocouples, used as wet and dry bulbs, and by measuring the SO_2 concentration with a Liston-Becker infrared gas analyzer. The flow rate through the fumigation chamber was 1 liter/minute for all tests except the temperature experiments, in which the rate was 600 ml/minute. No difference in SO_2 toxicity was observed due to change in flow rate.

After fumigation, the patches were inverted momentarily on the surface of cold gelatin in petri plates. The spores adhered to the gelatin in the same relative positions occupied during treatment. No nutrient was added to the gelatin, and the pH was about 6.

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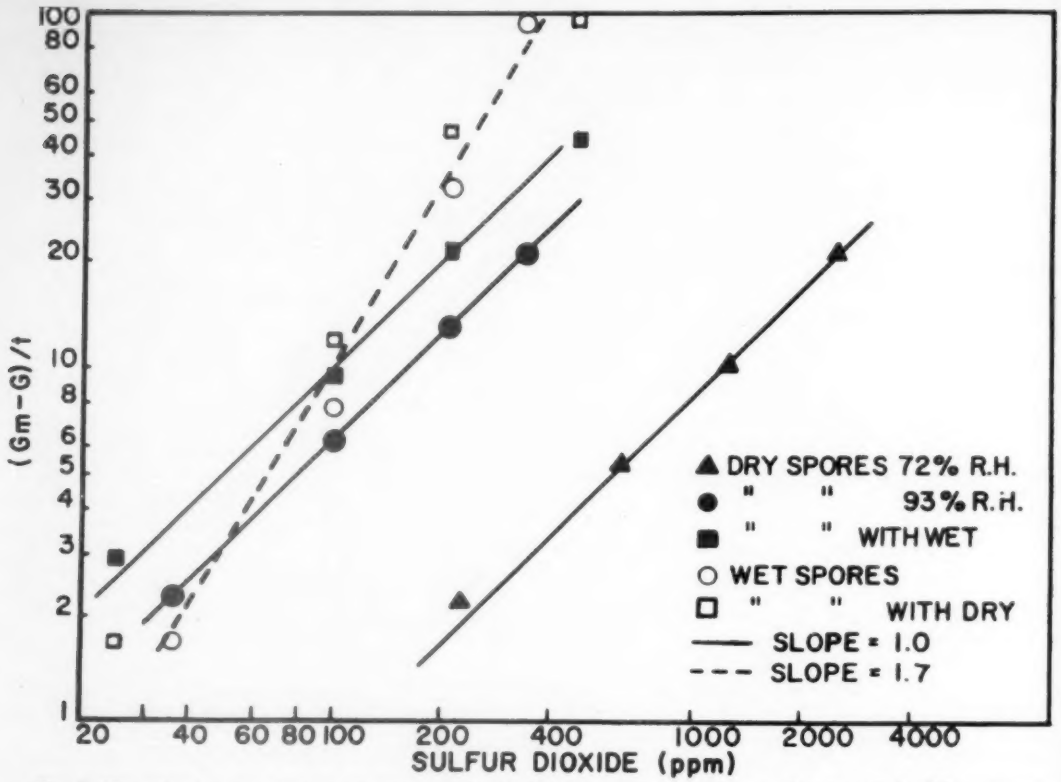

Fig. 1. The reduction in percentage of spore germination/minute exposure to SO_2 as a function of the SO_2 concentration on logarithmic coordinates at 20°C. Wet paper in the fumigation chamber increased relative humidity to nearly 100%.

The spores were incubated at 20°C for about 20 hours. Then, total spores and germinated spores were counted in 5 microscope fields (× 430), each containing 20-25 spores, and percentage of germination was calculated.

Each experiment was replicated 3-6 times, depending on the variability of the results. Data were analyzed statistically. Probability levels exceeding 99% were considered significant.

The following equation relating spore germination to SO_2 concentration and exposure time was used to condense and summarize the data: equation 1, $Go-G = k\,C^n t$, where Go = percentage of germination of untreated spores, G = percentage of germination of treated spores, C = concentration of SO_2, t = exposure time, and k = a proportionality constant.

The equation may be rearranged and logarithms of each side taken as follows: equation 2, $\log Go-G/t = \log k + n \log C$. The value of n was determined from the slope formed by plotting $\log Go-G/t$ *vs.* $\log C$ (Fig. 1.) After determining n, k was calculated directly. This equation simply states that the reduction in germination is proportional to the effective dose ($C^n t$) of SO_2. At any given concentration of SO_2, equation 1 is formally identical with the lower terms of a polynomial and the linearity of the response to exposure time was tested statistically using the polynomial method (5). The calculated response and the experimental points also may be compared (Fig. 2).

RESULTS.—*SO_2 concentration and exposure time.*—The toxicity of SO_2 depended on the concentration and the exposure time. When exposure time and environmental conditions were constant, germination was a function of SO_2 concentration. When the spores were dry during treatment, germination was a linear function of SO_2 concentration and the value of n was 1.0. If spores were wet, germination became a power function of the concentration and the value of n was 1.7 (Fig. 1, 2). This effect of water on the value of n cannot be attributed to a change in concentration of SO_2 due to absorption by the wet paper. When wet spores on wet paper and dry spores on polyethylene were placed side by side on a microscope slide, and exposed to SO_2 gas, the respective values of n were almost identical to those obtained in the previous separate experiments (Fig. 1). Therefore, this change in n must be due to the water immediately surrounding the spore or to the effect of water on the physiology of the spore.

The graphs (Fig. 2, 3) and the statistical treat-

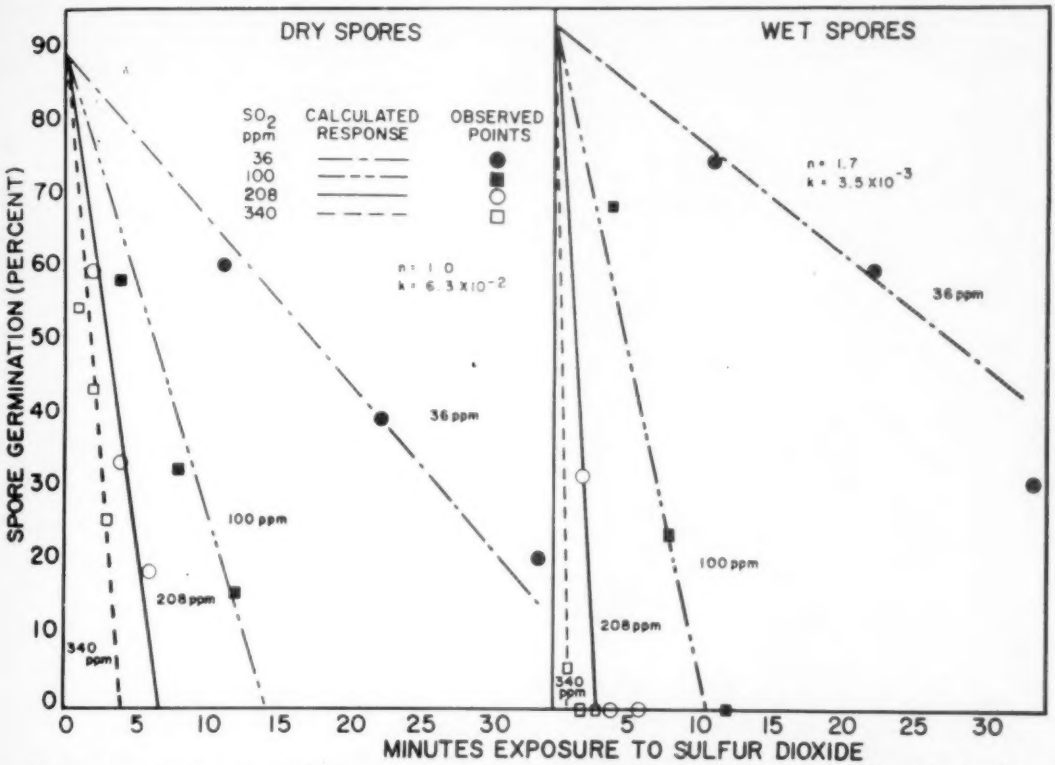

Fig. 2. Spore germination as a function of SO_2 concentration and exposure time at 20°C and 96% relative humidity. Dry spore treatment means less than 69% and wet spore treatment means less than 72% are significantly different from the checks (90% and 94%, respectively).

ment showed that germination decreased linearly with increased time of exposure to SO_2.

Relative humidity and temperature.—Relative humidity had a great influence on the toxicity of SO_2 to dry spores. A concentration of 100 ppm SO_2 for 12 minutes was very effective at 96% RH, but had no significant effect on germination at 75% RH. Since a wide range of relative humidity could not be studied effectively at any single dose of SO_2, the desired comparisons were based on values of k assuming that $n = 1$ for dry spores at any relative humidity (Fig. 1). The toxicity of SO_2 at 96% RH was 23 times as great as at 75% RH and at least 11,000 times as great as at 0% RH (Table 1). The k values for wet and dry spores cannot be compared directly because of differences in the exponent n. Based on the value of k, the toxicity of SO_2 also increased about 1.5 times for each 10° rise in temperature from 1° to 30°C (Fig. 3).

Since relative humidity at any given vapor pressure is dependent on temperature, the combined effect of temperature and relative humidity must be considered. The data clearly show that k is not a function of vapor pressure (Fig. 4). When the vapor pressure was lowered by reducing the temperature at a constant relative humidity, much less effect on k was observed than by reducing the relative humidity at a constant temperature.

TABLE 1. Germination of *Botrytis cinerea* spores after exposure to different concentrations of SO_2 gas while dry at indicated relative humidities at 20°C: means less than 69% are significantly different from the checks

Relative humidity (%)	SO_2 (ppm)	% germination after exposure for indicated time (min.)[a] 0	4	8	12	16	24	60	k^c
–	0	93							
93 – 96	100		66	39	24				6.6×10^{-2}
87 – 90	520		60	20	1				1.6×10^{-2}
73 – 75	2100		64	41	30				2.8×10^{-3}
58 – 60	3400		72	69	55				9.3×10^{-4}
39 – 40	5100		89	81	69				3.8×10^{-4}
19 – 20	6800			87		88[b]	91[b]		3.1×10^{-5}
0	8500			91		92[b]	88	90	5.9×10^{-6}

[a] From 5 replications, 2 check plates/replicate.
[b] One missing value.
[c] $k = \dfrac{Go - G}{C^n t}$, $n = 1$. See text.

DISCUSSION.—These data provide some indication of the mechanism of the action of SO_2 on *Botrytis* spores. The effect of relative humidity on SO_2 toxicity indicates that the hydrated form of SO_2 (sulfurous acid) is the active agent. Sulfurous acid is a more effective fungicide than its salts (3). Since sulfurous acid is

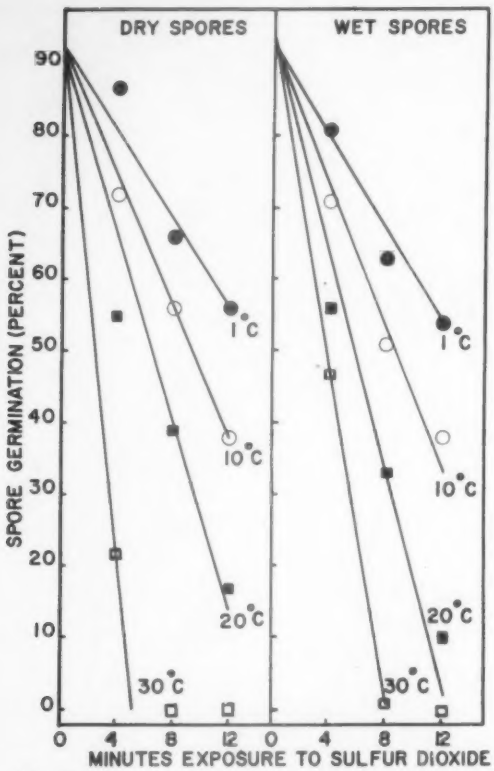

Fig. 3. Spore germination as a function of temperature and time of exposure to 100 ppm SO_2 at 96% relative humidity. Treatment means less than 81% are significantly different from the checks.

highly reactive, it may inhibit spore germination by combining directly with essential cell constituents. The failure of the spores to recover from SO_2 inhibition after they are removed from the SO_2 atmosphere may be attributed to an essentially irreversible combination of the sulfurous acid with the cellular material. The fact that SO_2 became more toxic as the temperature was increased and that spore germination was reduced linearly as the exposure time was increased supports this view (4). The small effect of temperature suggests that physical processes such as the hydration of SO_2 or the diffusion of the sulfurous acid to sensitive sites in the cell limit the rate of reaction (6).

Cant and Nelson (2) suggested that decay control by SO_2 is a function of the SO_2 "concentration-time factor" (the dose or Ct). If $n = 1$ for this process, good correlation between dose and effect may be expected. If $n \neq 1$, the more general quantity $(c^n t)$ or "effective dose" should be substituted and may prove equally useful. If the value of n is not known from experiment, dosage calculations should be used with caution.

In these experiments, decay control was not considered. If the control of gray mold of grapes is a function of the ability of SO_2 to inhibit spore germination, however, the temperature and relative humidity during treatment may greatly influence the effectiveness of fumigation. A small decrease in temperature may increase the relative humidity and the net effectiveness of the treatment, or warm fruit in cool air may lower the relative humidity surrounding the fruit and decrease the effect of the SO_2 treatment. Data correlating spore germination and infectivity under various controlled conditions are needed. Additional observations of temperature and relative humidity under commercial conditions may indicate whether fumigation practices could be improved by better control of environmental conditions.

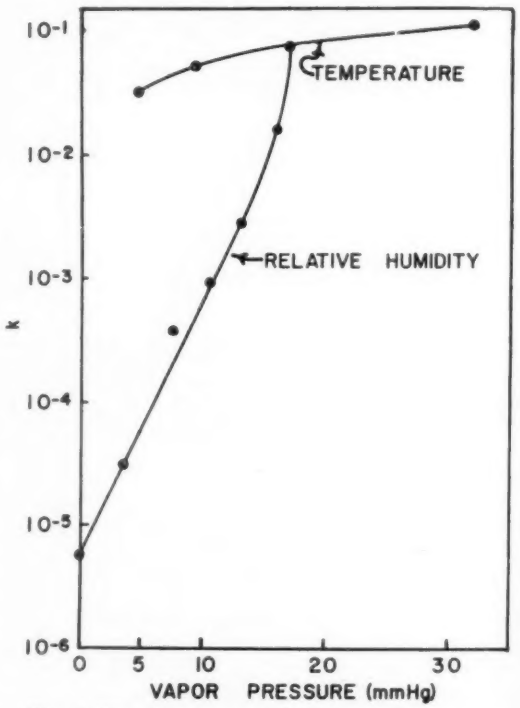

Fig. 4. The value of k as a function of vapor pressure. The vapor pressure was varied by a change in temperature from 1° to 30°C at constant relative humidity or by changing the relative humidity from 0% to 96% at constant temperature. The data are from Fig. 3 and Table 1. Logarithmic scale was used only for convenience.

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THE PATHOGENIC RELATIONSHIP OF CLITOCYBE TABESCENS TO PEACH TREES

Donald H. Petersen

Department of Botany and Bacteriology, Clemson College, South Carolina.

Cooperative investigations of the Crops Research Division, ARS, U.S.D.A. and the South Carolina Agricultural Experiment Station. Approved by the Director, South Carolina Agricultural Experiment Station, as Technical Contribution No. 351.

Plant Pathologist, Crops Research Division, ARS, U.S.D.A. Grateful acknowledgement is made of the help of E. James Koch in analyzing the data.

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SUMMARY

Seedling peach trees were grown in pots of sterilized soil containing oak stems inoculated with pure cultures of *Clitocybe tabescens*. No infection occurred during 10 months of continuous growth in which the peach roots remained in direct contact with the inoculum. In a field experiment, June-budded nursery trees were planted over peach wood infested with *C. tabescens*. After 4 years, localized infection occurred in 1 or 2 roots on 8 of 19 trees. There were no above-ground symptoms of disease. On the basis of these results, it was concluded that the association of *C. tabescens* with the dead roots of dying or dead young trees planted in previous peach tree sites reflects a saprophytic role for the fungus rather than parasitic. Eventual decline of peach trees due to infection by this fungus is probable, but it may require several years.

INTRODUCTION.—Over a period of years, Rhoads (4, 7, 9) observed that *Clitocybe tabescens* (Scop. ex Fr.) Bres. was associated with many species of dead or dying trees, including peach. Based on these observations and inoculation of Australian pine (5, 6), he concluded that *C. tabescens* was a destructive pathogen. Few controlled inoculation tests have been conducted, however, to prove its pathogenicity. Plakidas (3) first reported successful inoculations using pure cultures of *C. tabescens*. He cultured the fungus on thick pieces of autoclaved pear and tung wood. The infested wood was placed below-ground in contact with wounds made on the main roots and trunk bases of yearling and 8-year-old pear trees. Of the 18 inoculated trees, only 2 showed above-ground symptoms of disease at the time of his report, about 40 months after inoculation. No other inoculation studies have been reported.

C. tabescens was first reported as the apparent cause of a peach root- and trunk-rot in South Carolina and Georgia by Savage, *et al.* (11) in 1952. These authors stated that the fungus was of widespread occurrence and destructive in peach orchards of the 2 states. They reported *Clitocybe* root-rot of peach trees mainly on land planted successively to peaches for 30-50 years. They concluded that the prevalence and wide distribution of this disease gave at least a partial answer to why peaches did not follow peaches successfully under Georgia conditions. Rhoads (8) and Savage and Cowart (10) made similar conclusions.

The above authors, in citing the destructive nature of *C. tabescens* to peach, apparently based their conclusions only on the association of the fungus with the dead roots and crown of dying or dead trees, since no pathogenicity studies on the host were reported. Therefore, the present study was undertaken to determine whether *C. tabescens* is able to penetrate living, uninjured peach roots and cause progressive decay and eventual death of trees.

PURE CULTURE PATHOGENICITY STUDY.—*Materials and methods.*—Seedling peach trees with injured or uninjured roots were planted in clay pots containing oak stems infested by *C. tabescens*. The original cultures of the fungus were obtained from the tissues of sporophores produced on peach wood. Six-8 pieces of a living stem of white oak (*Quercus alba* L.), 0.75-1.0 in. in diameter and 4 in. long, were placed upright in each of several 1-gal, wide-mouthed, glass jars. One-half cup of corn meal and 200 ml of distilled water were added to each jar and the units were steam-sterilized. After sterilization, the wood and corn meal in each jar were seeded with 3 malt agar (Difco) slant cultures of the fungus and the jars capped with several layers of sterile cheesecloth. The

seeding was accomplished while the jars remained in the autoclave with the steam line open. These cultures were incubated in a dark humid room for 3 months at 28°C prior to use as inoculum. The cheesecloth jar covers were kept moist with distilled water during the incubation period.

For a seedling source, after-ripened peach seeds were planted individually in 4-in. clay pots of sterilized soil. When the seedlings reached a height of 4-6 in., the soil and plant were carefully removed from each pot and the soil washed from the roots. The seedlings then were planted in contact with the prepared inoculum by placing sterilized soil in the bottom of 6-in. clay pots, laying a piece of infested wood on the soil in each pot, placing the seedling roots in contact with the wood, and filling the pots with sterilized soil. Ten pots of oak wood inoculum were planted with uninjured seedlings and 10 with injured seedlings. Plants were injured by severe root pruning and by the removal of a piece of bark from the stem below the soil line. A control set of plantings was made using oak stem media. The seedlings were planted in contact with the inoculum in April and were placed on an outdoor, partially shaded bench until October. During this period, the pots were surrounded by sterilized sawdust to prevent rapid drying and to aid in lowering the temperature of their contents. Water was supplied by an overhead sprinkler system. In October, the pots were moved into a greenhouse; they were dug in February.

The preparation and use of the oak stem medium were suggested by Plakidas (3) in his demonstration of pathogenicity of *C. tabescens* to pear trees. This method of inoculation also has proven useful in demonstrating pathogenicity of another root-rot species (*Armillaria mellea* (Vahl ex Fr.) Quel). Bliss (1) reported that Lovell peach seedlings were severely infected within 240 days after they were planted in soil containing infested citrus root segments. The present author has noted infection of seedling peach roots by *A. mellea* within 3 months after placing the roots in contact with infested pieces of peach and oak stems placed in pots of sterilized soil. Thus, it appeared that if *C. tabescens* could cause the death of peach trees during their first season in an orchard, as was reported by Rhoads (8), this method should demonstrate its virulence under controlled conditions.

Results.—After 10-months' continuous growth in contact with the inoculum, all trees were living except 3 which had been girdled by peach tree borers (*Aegeria exitiosa* Say). Examination of the roots and lower stems revealed no evidence of infection by *C. tabescens*, although the seedling roots were planted in direct contact with the inoculum. The fungus was isolated from 17 of 20 pieces of oak stems used as inoculum. A single sporophore of *C. tabescens* was noted in 1 of 17 pots in mid-November.

FIELD PATHOGENICITY STUDY.—*Materials and methods.*—Death of roots from various unknown causes is characteristic on young peach trees planted in old peach-orchard or peach-tree sites. It also is well known that *C. tabescens* and other fungi associated with root decay are particularly adapted to saprophytic existence. Thus, in order to avoid complications normally associated with replanted sites, this experiment was established on land not previously planted with peach trees or other hosts. A location on the Experiment Station farm at Clemson, S.C., was chosen because there had been no host plants for at least 26 years. The soil was Cecil clay loam, a common soil type for peach orchards in northern Georgia and South Carolina. The site is within the geographic range of the fungus, which has been noted on peach and oak nearby.

Peach nursery stock was planted over peach wood naturally infested with *C. tabescens* and over wood free of the fungus. A randomized-block experimental design with 10 replicates was used for the 7 treatments. The inoculum for treatment numbers 5, 6, and 7 consisted, respectively, of *C. tabescens*-infested peach stumps, infested peach roots, and 1 bu of soil and debris from under the crowns of *C. tabescens*-infested peach trees. In a comparable set of treatments, numbers 2, 3, and 4, the material was obtained from and under 7-year-old healthy peach trees. In treatment 1, trees were planted in the normal manner with no material under them.

The *C. tabescens*-infested material was obtained from peach orchards near Fort Valley, Ga., and Columbia, S.C. The presence and identity of the fungus were established by sporophores produced on the infested wood. Noninfested materials were obtained from Clemson, S.C., using only those stumps and roots that apparently were free from decay, regardless of cause. Soil and debris for treatment 4 were taken from under trees free from decay.

All wood and soil materials were deposited during November, 1955. Initially, 6 in. of the experimental site soil were between the top of the deposited material and the roots of the young trees. This 6-in. soil barrier allowed the injuries, which occurred during the digging of the nursery trees and their transplanting, to heal before their roots came in contact with the inoculum. Trees of the Keystone variety, June-budded on seedling stock, were secured from a central Tennessee nursery free from *C. tabescens*. They were transplanted in January, 1956.

During the 4 years of the experiment, the orchard was managed according to recommended horticultural practices. Peach insect and disease control programs were carried out. In addition, the trees were mulched with hay or straw each growing season. Between September 15 and November 15, when the sporophores of *C. tabescens* might be expected, the soil around all trees was kept moist.

Annual increases in trunk diameter of the trees were used to measure effects of treatments on tree growth. All trees were caliper-measured at planting and annually thereafter at a point approximately 1 ft above the soil line. In January, 1960, the trees were dug and the roots and trunks examined for infection by *C. tabescens*. Each tree was removed so

TABLE 1. Mean accumulative trunk diameter of peach trees planted over *C. tabescens*-infested material, over noninfested material, and of trees planted normally

Treatment	Mean accumulative growth in mm and D.M.R.[a]							
	1956	.05	1957	.05	1958	.05	1959	.05
1. Normal planting	34.0	a	61.1	a	87.6	a	111.0	c
2. Over noninfested stumps	38.3	b	69.2	ab	93.9	ab	118.5	abc
3. Over noninfested roots	39.2	b	67.0	ab	92.2	ab	114.5	ab
4. Over noninfested debris	40.0	b	68.1	ab	95.2	abc	119.0	abc
5. Over infested stumps	41.0	b	73.0	b	99.6	bc	125.5	cd
6. Over infested roots	39.4	b	70.4	b	96.4	bc	121.0	bcd
7. Over infested debris	40.0	b	72.2	b	102.8	c	128.8	d

[a]Duncan Multiple Range test at 5% level. Treatments having the same letter are not significantly different.

that all but the smallest roots in a 2-ft radius from the trunk remained intact. The ball of soil lifted with the trees was washed from the roots, so that the roots could be examined critically.

Results.—There was no apparent deteriorative effect on the growth of trees planted over the *C. tabescens*-infested material as measured by trunk diameter (Table 1). Also, there was no evidence of the fungus on the above-ground portion of the trees during the 4 years of the experiment.

Trees planted in the normal manner probably were smaller because the holes for these trees were only of sufficient size and depth to receive the roots. They did not equal the smallest holes receiving the foreign material. The fact that the trunks of the trees planted over the *C. tabescens*-infested material were larger is not biologically significant in peach production practices. Nothing in this study indicated the factors involved in the larger size of the trees.

Five of the 9 trees (1 was broken off by a tractor in 1957) planted over infested stumps and 3 of the 10 trees planted over infested roots were diseased at the end of the fourth growing season. *C tabescens* was isolated from the roots of all 8 diseased trees and identified by cultural characteristics. In each case, infection was confined to 1 or 2 roots directly under the crown of the tree. Infected roots ranged from 0.5-1.5 in. in diameter; the apparent length of the infected area in no case exceeded 8 in. The position of the infected roots clearly indicated that they had been in direct contact with the inoculum.

In most instances, the bark of the roots in the infected areas was still intact; there was no evidence of external injury to the roots prior to penetration by the hyphae of *C. tabescens*. Callus tissue was absent, indicating that the fungus was actively causing decay, however slowly, of the root tissues. No symptoms were evident in the above-ground appearance of the trees to indicate that infection had taken place nor was there any appreciable difference in the amount of growth in any year, as determined by trunk diameter.

The fungus was recovered from each tree site where infested stumps and roots had been deposited. The organism was not found in the roots of any trees planted over material from healthy peach trees, over either type of soil and debris, or in the roots of those trees planted normally.

All reported symptoms of root rot caused by *C. tabescens* have been based on observations of advanced stages of decay following tree decline or death. The present work afforded an opportunity to study the symptoms as they appeared on peach roots in earlier stages of the disease and before visible symptoms appeared on the above-ground portions of the tree. It is not known how long the fungus had been present in the roots prior to the pulling of the trees. Externally, the infected areas of the roots were slightly sunken and appeared grayish-black, in sharp contrast to the color of vigorous living roots. Under the bark, the mycelial mat of *C. tabescens* was well developed to within 1.0-1.5 in. of the margin of the diseased tissue. All tissues under the mycelial mat were somewhat darker than normal and later became similar in color to the exterior bark. Even at this stage of decay, the wood tissues remained firm, but appeared water-soaked. In a further progression of the decay, the mycelium penetrated throughout the root tissues advancing from the mat under the bark to the centers of the roots and outward into the bark.

Tissue discoloration extended a short distance ahead of the mycelial mat and was greatest in the cambium. No hyphae were found in isolations or differentially stained tissue sections at the outermost limits of the discoloration, indicating that *C. tabescens* causes host cell changes in advance of its penetration. The hyphae of the fungus apparently grow up or down the root tissues from the point of infection with equal facility. No rhizomorphic extrusions to the outside of the bark were noted and there was no evidence of rhizomorphs on the bark.

The field experiment was not designed to include the effect of host predisposition on disease initiation and development. Over the 4-year period of the experiment, however, high and low extremes of temperature and rainfall occurred several times.

DISCUSSION.—Orchard observations by various workers have shown that the longevity of peach trees planted in former peach-orchard or peach-tree sites is often considerably less than that of the original planting, a condition often termed the "peach replant problem." *C. tabescens* may be found associated with the dead roots on these dead or dying trees. Savage, *et al.* (11) reported that young trees were attacked by this fungus when they were planted in a previous

orchard site. Rhoads (8) noted the presence of the fungus on young trees that died prior to the end of their first growing season in a previous South Carolina orchard site. In all cases where *C. tabescens* has been associated with young trees, the trees were on land previously planted with peach.

The death of young trees on previous orchard sites has been commonly observed, however, in the absence of this fungus. In 1 such site near Columbia, S.C., in which 240 trees were set out by the author, 76 died by the end of the first growing season. None was infected by *C. tabescens* even though the fungus was present in a few trees of the original planting which were 11 years old at the time they were pulled.

Owen, *et al.* (2) reported that the fungus may attack trees in original orchard sites. They found the fungus in association with many 20-year-old trees in an orchard near Watkinsville, Ga. Savage, *et al.* (11) and Rhoads (8) mentioned several observations of the fungus in association with old trees on original orchard sites. During the past 7 years, the author has identified *C. tabescens* by its sporophores or by cultural characteristics in several instances where it was associated with older dying or dead trees planted on original sites.

During the present work on *C. tabescens*, the author found the organism most frequently associated with trees older than 10 years on original sites or associated with young trees planted in previous orchard or tree sites. This apparent inconsistency in the time of association of the fungus with dead or dying trees on original or previous sites was well brought out, but apparently unrecognized, by Savage, *et al.* (11) and by Savage and Cowart (10). In a study on the factors involved in the failure of peach trees to follow peach trees successfully, they pulled the trees of a 17-year-old orchard and replanted it. A portion of their report (11) stated:

"The experimental planting at the Georgia Experiment Station made in 1946 included 98 original plot trees. Of this number 53 had died by September of 1952. Of these dead trees about 50 percent died as a result of *Clitocybe tabescens*, and it would appear that many more died of the same cause although it was not recognized at the time of death. It is interesting to note that at the end of 17 years 95 percent of the trees in the 1929 orchard planted on this site were still living. This is in marked contrast to the 54 percent of the trees dead after six years in the second orchard planted on this site."

A build-up of inoculum in the original orchards may account for the early attack by the fungus on trees planted on old sites (8, 10, 11). This conclusion, however, does not account for the longer life of the original trees, since the inoculum must have been present in close association with at least some of the trees for varying periods of their life in the orchard. Rhoads (8) pointed out, and the writer concurs, that infection takes place at the point of host-root contact with infested wood in which the fungus lives saprophytically. Since the initial point of infection was well under the soil surface in all observed instances, it appears that root contact with the inoculum is the only natural means of infection.

There must be present, in old orchard sites, certain factors, other than the amount of inoculum of *C. tabescens*, which favor the early death of replanted peach trees. Little is known about the agencies involved, or their interactions, in the so-called peach replant problem. A common result of their presence, however, is the death of roots of young trees when they are planted in previous orchard or tree sites. The possibility of increased populations of other microorganisms in old peach orchard soils is recognized. As a group, their effect on peach tree longevity has not been studied. Consideration also should be given to the part they may have in affording places of entry for hyphae of *C. tabescens*.

C. tabescens can become well established in sections cut from living peach roots in 3 months or less. The fungus has been isolated from old peach roots buried in the soil as long as 6 years after the trees were removed. These facts suggest that the fungus is well adapted to a saprophytic existence. The studies reported herein showed that no infection occurred on living peach roots exposed to direct contact with infested oak stems for 10 months. Only a small amount of infection occurred when field-grown trees were exposed to infested peach trunks and roots for 4 years. In both studies, the soils had not been previously planted to peach. Thus, it would appear that the association of *C. tabescens* on young trees planted on previous sites can be attributed to the rapid saprophytic establishment of the fungus on their characteristic dead roots. Whether these young trees die as a result of attack by *C. tabescens* or as a result of the unknown factors involved in the peach replant problem cannot be determined in the field since the association of the fungus with dead or dying young trees on replanted sites is not necessarily indicative of a pathogenic relationship. In fact, the results of the above studies would indicate that the fungus may only be invading the dead root tissues caused by the unknown factors of the peach replant problem. Certainly, the infested dead roots on the trees would serve as a source of abundant inoculum for an eventual attack on the healthy roots.

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KINETIC STUDY OF THE HEAT INACTIVATION OF TOBACCO MOSAIC VIRUS INFECTED CENTERS AND POTENTIALLY INFECTIBLE SITES ON NICOTIANA GLUTINOSA

Jia-Hsi Wu and Irving Rappaport

Department of Botany, University of California, Los Angeles.

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SUMMARY

The kinetics of inactivation by heat of infected centers (host-virus complexes) were different from those reported for whole virus or infectious nucleic acid *in vitro*. Both the infected centers, immediately after inoculation, and the potentially infectible sites were sensitive to elevated temperatures. They were rapidly inactivated at temperatures where intact virus and infectious nucleic acid remained stable. Initially, the heat sensitivity of infected centers was equal to that of the potentially infectible sites. Within 3-4 hours after inoculation, however, this sensitivity diminished to essentially 0 with the heat doses used in these experiments.

The heats of activation for inactivation of infected centers immediately after inoculation and for infectible sites were calculated. A value of approximately 74,000 cal/mol for both was obtained for the temperature range of 43-49°C. This value suggests that the heat inactivation of infected centers and infectible sites probably involves the denaturation of proteinacious substances of the host cell, and is independent of the presence of any infectious unit.

INTRODUCTION.—The effect of temperature on host-virus complexes has been the subject of a number of investigations. Since a thorough review of the earlier work has been published by Kassanis (4), only the more recent pertinent studies will be mentioned here. Yarwood (10), investigating the effect of heat on Pinto bean leaves inoculated with tobacco mosaic virus (TMV), found an exponentia' decline in the number of lesions appearing following treatment for different periods at 50°C. More striking, however, was that a 5-10 hour delay in the heat treatment after inoculation caused a large increase in the absolute number of lesions. Yarwood attributed this to an activation of TMV infections that would not have progressed to lesion formation without the heat stimulus. In extending some of these studies, the exponential decline in lesion number as a function of heat dose was reinvestigated to ascertain whether the effect of heat was on the virus, or on some constituent(s) of the host necessary for the virus infection to proceed to the point of lesion appearance.

The kinetics of heat inactivation of whole virus (6) and infectious nucleic acid (3) *in vitro* are well established. What has not previously received a thorough investigation, however, is a kinetic study of heat inactivation of the infected center (host-virus complex). It was the purpose of this investigation to compare such data with those for the heat inactivation of the *in vitro* virus and infectious nucleic acid.

MATERIALS AND METHODS.—*Nicotiana glutinosa* L. leaves were detached from plants either before or after inoculation with 5×10^{-4} mg/ml of TMV strain U1 in phosphate buffer (pH 7.0) containing 50 mg/ml celite. Inoculated leaves then were washed with tap water to remove the celite and excess inoculum from leaf surface. For the heat treatment, a water bath was used, regulated with a thermostat. Leaves were detached from the plants and half of each leaf was inserted through a narrow slit in a 0.25-in. thick sheet of "Styrofoam" plastic which floated on the water. Thus, half of the leaf was submerged while the other half remained exposed to the air (Fig. 1). The air

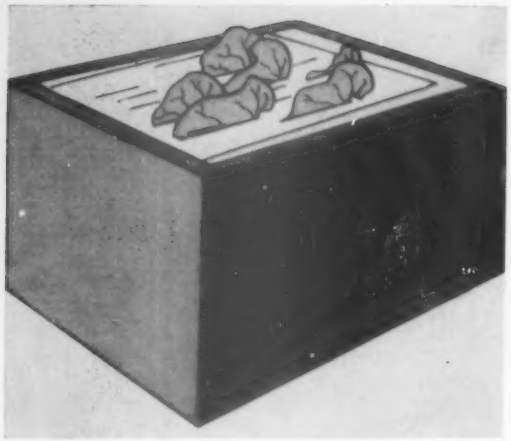

Fig. 1. *Nicotiana glutinosa* leaves supported by a sheet of "Styrofoam" floating in a thermostatically controlled water bath. Half of each leaf is submerged; the other half is exposed to the air.

temperature above the plastic plate was not significantly different from the air temperature outside of the water bath during the heat treatment. The number of lesions produced on the heated side of the leaves was always compared with the unheated opposite half which served as a control. After heat treatment, the leaves were removed, and their petioles inserted through a small hole in another "Styrofoam" plate which floated on water in a roasting pan. The pans were covered with a glass plate and placed under continuous illumination (*ca.* 500 ft-c) at 20-22°C (7). The lesions appearing were counted 4-5 days after inoculation.

EXPERIMENTAL RESULTS.—Since Yarwood (9) reported that water had a deleterious effect on infected centers on Pinto bean, the effect of dipping inoculated *N. glutinosa* leaves in water for different durations of time was first investigated. For example, in 1 of several such experiments, inoculated *N. glutinosa* leaves were washed with tap water as described above. Then, half of the leaves were immersed for as long as 8 minutes in an unheated water bath kept at room temperature (27°C). This length of time was equal to the longest treatment employed in subsequent experiments. Twenty half-leaves immersed in water produced 306 lesions, compared with 299 lesions produced on the opposite 20 half-leaves which were exposed to the air. Thus, with *N. glutinosa*, a water immersion for as long as 8 minutes did not affect the number of lesions produced. Therefore, the reduction in lesion number arising from the hot water treatment described here is interpreted as an effect associated with elevated temperatures and not with the effect of water as such.

Inactivation of TMV-infected centers by heat.—A number of *N. glutinosa* leaves were inoculated with

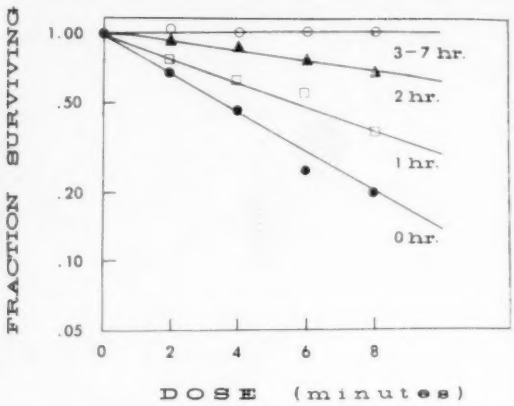

Fig. 2. Heat survival curves of TMV strain U1 infected centers at different times after inoculation. The number of hours indicates the time between inoculation and exposure to heat at 43°C. The points indicate the ratio of the number of lesions on heat-treated half-leaves to the number of lesions on the nontreated, opposite half-leaves.

5×10^{-4} mg/ml of the common strain of TMV, U1. The excess inoculum was removed by washing the leaves with water. In some experiments, the leaves were treated immediately with heat (43°C); in others, the heat treatment was delayed for 1, 2, 3, or 7-8 hours after inoculation. The experiments were repeated several times. A summary of the data is plotted in Fig. 2.

In confirmation of Yarwood (10), the data suggest that the infected centers are inactivated exponentially by heat. The greatest rate of inactivation was measured initially after inoculation. As time after inoculation

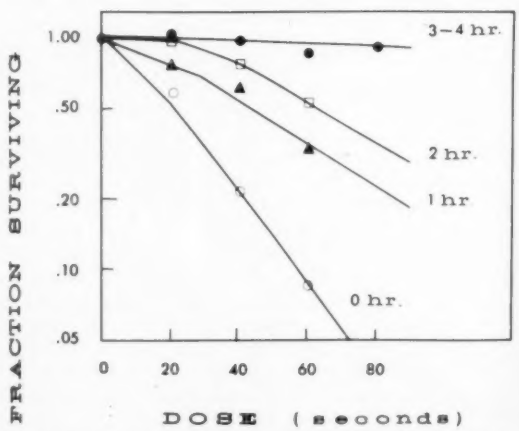

Fig. 3. Heat survival curves of TMV strain U1 infected centers at different times after inoculation. The number of hours indicates the time between inoculation and exposure to heat at 49°C. The points indicate the ratio of number of lesions on heat-treated half-leaves to the number of lesions on the nontreated, opposite half-leaves.

increased, the rates declined. When the heat treatment was delayed beyond 3 hours, no measurable loss in lesion number was apparent. The experiment was repeated using a heat treatment of 49°C (Fig. 3).

Again, the inactivation curves immediately after inoculation were exponential, although much steeper. Infection, apparently, was extremely sensitive to heat. By comparison, intact virus, and infectious nucleic acid *in vitro* are inactivated only slightly at temperatures of 43-49°C and short heating periods (3, 6). This large difference between the *in vitro* sensitivity to heat of the infectious units and the infected centers suggests that the host plays a dominant role in determining the outcome of these experiments. The following experiments were designed to assess the host's contribution to the over-all reaction rates.

Inactivation of potentially infectible sites by heat.— Using the method of Furumoto (2), infectible sites were first created by brushing both halves of *N. glutinosa* leaves with a celite suspension (50 mg/ml) in pH 7.0 phosphate buffer solution. The leaves were immediately rinsed with water. Then, half of the leaves were submerged in a water bath either at 43° or 49°C for different periods. Immediately (within 1 minute) after the heat treatment, the entire leaves were dipped for 2-3 minutes in 1.0 mg/ml virus solution to produce maximum infection of those sites which remain infectible by TMV. The excess virus was washed off, and the leaves were incubated at 20-22°C until lesions appeared. The maximum interval of time between the actual creation of sites and their subsequent infection was 10 minutes. As before, the lesions were counted and the ratios of lesions between the heated and unheated half leaves were calculated. The results of several experiments are shown in Fig. 4.

It is evident that potentially infectible sites also are inactivated exponentially by heat. When the results for the infectible sites are compared with the previous results for infected centers, the infected centers

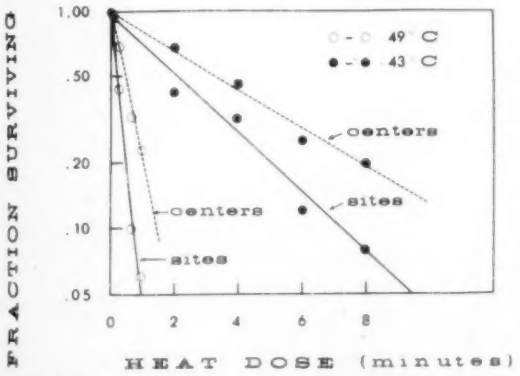

Fig. 4. Heat survival curves of potentially infectible sites and U1–TMV–infected centers at 2 temperatures. Both infected centers and infectible sites were treated with heat immediately after their creation.

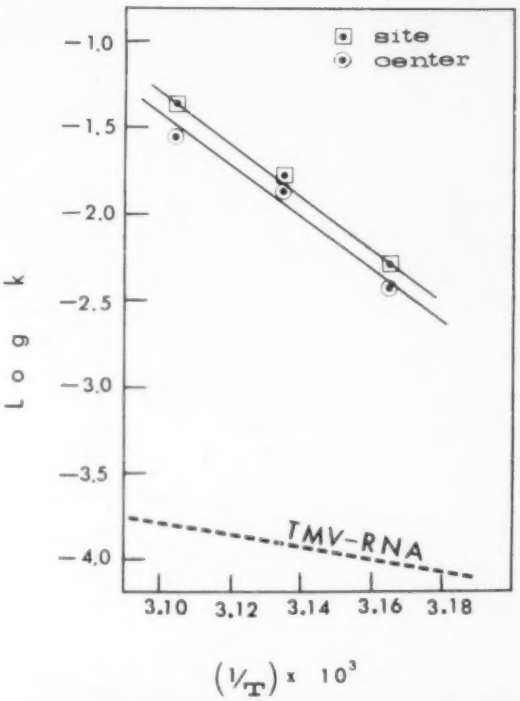

Fig. 5. Temperature dependence of the rate of inactivation of TMV–infected centers and the potentially infectible sites at 0 time. The heat inactivation of infectious nucleic acid *in vitro* is taken from Ginoza (3).

appear slightly more resistant to heat than the newly created infectible sites. This difference in the inactivation rates, however, may be attributed to the slightly different experimental design. Furumoto (2) has shown a loss in the number of infectible sites with time after their production by abrasion. This loss, which is rapid, was therefore added to that resulting from heat inactivation. Furthermore, it was noted that if abraded leaves were dipped into a virus suspension, and immediately given a heat treatment so as to minimize the time elapsed between inoculation and heating, the slopes of the inactivation curves for the infected centers approximated those of the infectible sites.

From the data shown in Fig. 4, and additional data obtained at 46°C, it was possible to estimate the heat of activation for inactivation of TMV potentially infectible sites as approximately 74,000 cal/mol for the temperature range 43-49°C. Fig. 5 shows a plot of the logarithm of the first-order rate constants of inactivation of potentially infectible sites at 0 time (within 10 minutes after creation) and for the infected centers (within 20 minutes after inoculation) *vs.* the reciprocal of the absolute temperature. These 2 lines appear straight and parallel.

For comparison, the heat inactivation data of infectious nucleic acid from TMV (3) also are included

in Fig. 5. The rate of inactivation of nucleic acid *in vitro* is a much slower process than that of either infected centers or infectible sites.

The intact virus is extremely resistant to inactivation at these temperatures and duration of heating. Price (5), using crude extracts of TMV infected plant juice, found essentially no difference in the number of lesions produced by a sample heated for 4 hours at 68°C and by its unheated control. In our own experience with purified TMV, a heat treatment of 30 minutes at 49°C resulted in no significant loss in infectivity.

DISCUSSION.—In contrast to Yarwood's (10) findings that the latent lesions in Pinto bean could be activated by a delayed heat treatment, a result also confirmed in this laboratory, no evidence of a similar phenomenon was obtained with *N. glutinosa* inoculated with U1 virus. A further difference noted between these 2 hosts was that heat-treated TMV lesions appearing on Pinto bean were larger than on nontreated plants, while similar heat treatment produced smaller lesions on *N. glutinosa*. Apparently, Pinto bean and *N. glutinosa* react differently to heat.

Within the temperature range of 43-49°C used for these *N. glutinosa* experiments, the effect of heat on the virus-infected centers, or the potentially infectible sites, follows first—order kinetics if the leaves are heated immediately after creation of the sites or infected centers. The calculated high value for the heat of activation of about 74,000 cal/mol for either infected centers or infectible sites suggests that a proteinacious material necessary for virus activity is being denatured (1, 3, 8). The fact that resistance to heat inactivation is acquired within 3-4 hours after inoculation of virus further suggests that the virus may either, a) stabilize the material which is at first sensitive to heat inactivation, or b) utilize this heat-sensitive material for only a brief time. Of course, increased resistance with time may be attributed to a healing process. The newly created infected centers and infectible sites would be most susceptible while the wounds were still open to the surface, but as time passed they would heal over.

It may be concluded, that the *in vivo* inactivation rate at the initial period of infection is a measure of the loss in cellular capacity to support a virus infection, and that the rate of inactivation is independent of the presence of virus. Since heat treatment primarily affects the leaf, resistance may vary according to the past history of the plant grown in the greenhouse. In general, we have found this to be true, for it was noticed that those plants grown at low light intensity were more sensitive to heat treatment and heat injury than those grown at higher light intensity. Furthermore, the young top leaves displayed a greater tolerance to heat than did the lower or older leaves.

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A BACTERIAL DISEASE OF PHILODENDRON

H. N. Miller and Lorne A. McFadden

Respectively, Plant Pathologist, Florida Agricultural Experiment Station, Gainesville; and Assistant Horticulturist, Sub-Tropical Experiment Station, Homestead.

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SUMMARY

A bacterial disease characterized by irregular, water-soaked areas on leaves and a collapse of petioles of philodendron was discovered in commercial nurseries. Favored by warm, moist conditions, the bacterium was pathogenic to several *Philodendron* spp., but not all. Morphological, cultural, and physiological characteristics and cross-inoculation studies indicate that the pathogen is a strain of *Erwinia chrysanthemi*.

Control of the disease was satisfactory with sprays of Agri-mycin, 100-200 ppm active ingredient, at 4-day intervals.

In 1952, the senior author observed a soft rot decay of leaves and petioles of *Philodendron selloum* Koch, causing economic losses in commercial nurseries in Apopka, Fla. Since then, the disease has been observed in several other species of large-leaved philodendrons and is now widespread in Florida in both greenhouse and lathhouse plantings. Repeated isolations made from diseased tissues revealed that bacteria were present in large numbers. One proved pathogenic to philodendron leaves.

The disease has been found in various areas in Florida and on plants shipped in from Central America. Except for brief reports by Miller (12, 13, 14), no references to the disease have been found in the literature.

Large numbers of philodendrons are now being propagated in Florida, from seed or cuttings. Young seedlings often become infected in seed flats or propagating beds, resulting in a rapid spread of the disease in the nursery. Philodendrons, like many other tropical foliage plants, are grown for their attractive ornamental foliage. The leaf rot organism often renders the plants unsalable, even though they are seldom completely blighted or killed.

Disease Symptoms.—The disease is first evident as small, irregular, water-soaked areas on the lower surface of the leaf blade (Fig. 1-A). Under optimum conditions, the spots enlarge rapidly; water-filled blisters often develop, followed by a total collapse of the leaf into a slimy, soft mass (Fig. 1-C). Under less favorable conditions, the lesions become dry and yellow to tan, with definite yellow margins. The pathogen may remain viable for extended periods in these dry lesions. With a change in conditions, the margins of the lesions again become water-soaked and the spots enlarge (Fig. 1-B).

The leaf stalks or petioles also may become infected. Spread of the disease into the petioles, however, is always from infected leaf blades. The petioles become water-soaked and mushy, and finally collapse. Progress of the disease is unusually rapid when these succulent tissues are attacked. Infected leaves and petioles may fall from the plant. The stems, crowns, and roots are never attacked.

Effects of Environment on the Disease.—Philodendrons are grown under lath or Saran shade structures, which are usually crowded, hot, humid, and poorly ventilated. Such an environment appears ideal for growth and development of the leaf rot organism. Outbreaks of the disease often coincide with periods of high temperature and high humidity or frequent rains. When philodendron plants are artificially inoculated and placed under conditions of high temperature and high humidity, infection is evident within 24 hours. When infected plants are removed to a cooler, less humid atmosphere, progress of the disease is markedly slowed down or temporarily halted.

Dissemination.—Spread of the pathogen in nurseries is primarily by leaf-to-leaf contact and by being spattered and washed about by rains or during watering. Seedlings pulled from infected seed flats may harbor the bacteria, resulting in later outbreaks of the disease within a planting. Certain pests, such as slugs, also are believed to carry the bacteria from infected to healthy plants.

Host-Range Studies.—In commercial nurseries, bacterial leaf rot is confined to large-leaved philodendrons. Natural infections have not been observed on small-leaved philodendron (*P. oxycardium* Schott.). (The spelling and authorities for plant names used were checked in Index Kewensis.) A host-range study was conducted whereby plants were artificially inoculated with the leaf rot organism and placed under optimum conditions for disease development. The following plants were found very susceptible: *P. selloum* Koch, *P. melinononi* Brongn., *P. squamiferum* Poepp. & Endl., *P. panduraeforme* Kunth; and a hybrid, *P. wendlandii* Schott × *P. laciniosum* Schott. Plants of *P. sodiroi* Hort., *P. laciniosum* Schott, *P. wendlandii* Schott, and *P. "hastatum"* (doubtful status) were moderately susceptible, whereas plants of *P. oxycardium* Schott, *P. andreanum* Devansaye, *Monstera friedrichsthalii* Schott, and *M. deliciosa* Liebm. were resistant or immune. Other species of tropical foliage plants, nonsusceptible when inoculated with the philodendron bacterium, include: *Dieffenbachia picta* Schott, *D. picta* var. *amoena* Hort., *Aglaonema modestum* Schott, *Pilea cadierei* Gagn. & Guill., *Peperomia obtusifolia* A. Dietr., *Schefflera actinophylla* Harms, *Syngonium podophyllum* Schott, *Scindapsus aureus* (Lind. & Andre) Engl., and *Alocasia* sp.

The Pathogen.—Six isolants of the bacterial pathogen causing leaf rot of philodendron were selected from 12 for further study. All cultures were purified 3 times by streaking them on nutrient agar and selecting single colonies. The cultures were maintained on Bacto-nutrient agar and the tests carried out at 30°C unless otherwise indicated. The pathogenicity of all 6 cultures was established by inoculating healthy plants of *P. selloum*. The procedures were essentially those suggested elsewhere (4). All carbohydrates used in the fermentation tests were sterilized by passage through sintered glass or Seitz filters. The concentration of each carbohydrate was 1%, unless otherwise stated; and of the organic acids, 0.15%. The reactions of all 6 isolants were similar. Since preliminary work suggested a resemblance to *E. chrysanthemi* Burk., *et al.* (2), cultures of this organism were included for comparison.

Morphology.—The pathogen was a nonspore-forming rod with rounded ends, occurring in chains, singly or in pairs. Cells from 24-hour cultures grown on Bacto-nutrient agar slants averaged .94 x 2.7μ (.79—1.0μ x 1.6—3.7μ), when measured in congo red smear preparations. The bacteria were motile and gram-negative, and exhibited peritrichous flagella when stained by the method suggested by Leifson (9).

Cultural characteristics.—Growth was good on 24-hour cultures on Bacto-nutrient agar. The colonies had a wavy margin, and were pale cream to grayish, and filiform with butyrous consistency. Growth was more luxuriant on freshly prepared potato-glucose agar on

Fig. 1. Bacterial leaf rot of philodendron. **A)** Early natural infection. **B)** Advanced infection. **C)** Advanced disease development, showing collapsed leaves and petioles. **D)** Left, philodendron leaf inoculated with the philodendron organism; right, philodendron leaf inoculated with *Erwinia chrysanthemi*.

which the colonies were cream to light tan, later turning brownish with wavy margins, and butyrous. Nutrient broth became turbid within 12–15 hours. A fine pellicle formed in 24 hours and floated in the medium. Clara's medium supported a moderate growth, whereas a heavy white growth developed in Fermi's solution in 24 hours. Litmus milk was reduced and coagulated to 2:1 curd and whey; the reaction was alkaline for about 10 days, changing to a weak acid in 3 weeks.

Growth on Endo agar was reddish, but the color of the medium remained unchanged. Deoxycholate agar supported good growth of pinkish colonies. There was good growth on Krumwiede's triple sugar agar; the medium turned yellow, and then pinkish or pale at the end of 1 week. Streaks on Bacto EMB (containing eosin, methylene blue, lactose, and sucrose) agar slants were dark violet, with a characteristic iridescent surface. Growth was slow, sometimes lacking, on bismuth sulfide agar. Colonies were dark brown at first, changing to almost black with a metallic sheen.

Physiological characteristics.—Growth of the philodendron pathogen in a 12% gelatin-nutrient medium at 20°C was slow to moderate, with liquefaction observed at 1 week. The medium was 0.5 liquefied after 1 month. When grown in 1% Bacto-tryptone broth, all isolants were indole positive when tested with either Kovacs or Ehrlich-Bohme reagents. The methyl red test was negative after 2 days, and acetylmethylcarbinol was produced after 48 hours as indicated by a positive Voges-Proskauer test. A positive nitrite test resulted when the pathogen was grown 24 hours in bacto-nitrate broth. A strong test for H_2S production was consistently observed on lead acetate paper suspended over tryptone broth. Growth was good in Ayers, Rupp, and Johnson medium containing ammonium salts and dextrose.

Growth was good on spirit blue agar containing cotton seed oil. The medium, beneath the colonies, became blue, indicating fat hydrolysis. When H_2O_2 was added to cells of the pathogen a strong test for catalase resulted. Growth in broth containing NaCl was moderate at 3% concentration, faint at 5%, and absent at 7%. Growth in Bacto-nutrient broth was evident within 1 week at 39° and 9°C, but not at 41° and 6°C. The thermal death point was 50–51°C.

Starch was not hydrolyzed, but a solidified sodium pectate medium was liquefied within 24 hours. The pathogen was urease negative. In shake cultures, all isolants were aerobic, with a few scattered colonies growing at the 20-mm depth. Growth and gas production were moderate to good in 10 days in lactose broth, and in 1 week in formate ricinoleate broth. A moderate turbidity, heavy sediment, and gas were produced in 1 week in Bacto-lauryl-tryptose-broth.

When grown in a synthetic medium containing sodium ammonium phosphate, monopotassium phosphate, magnesium sulfate, and 0.1% Bacto-yeast-extract and bromthymol blue, the pathogen produced acid and gas within 1 week from the following carbohydrates: arabinose, rhamnose, xylose, glucose, fructose, galactose, mannose, sucrose, melibiose, 0.5% celobiose, 0.5% salicin, and mannitol. Acid and gas were produced in lactose after a 10-day incubation. Raffinose turned acid in 3 days, and gas formed in 2 weeks. Trehalose, inulin, dulcitol, 0.5% melezitose, and alpha-methylglucoside were not utilized in 3 weeks. Acid, but no gas, formed in media containing dextrin (trace after 3 weeks), glycerol (5 days), maltose (1 week), sorbitol (1 week), and 5% ethyl alcohol (3 weeks). There was an alkaline reaction in media containing 0.15% each of sodium citrate, sodium malonate, and sodium tartrate. Sodium hippurate was not utilized in 1 month.

Sensitivity to antibiotics.—To test the sensitivity of the philodendron pathogen to selected antibiotics, Bacto sensitivity discs were placed on seeded agar plates of Bacto heart infusion agar and incubated 24 hours at 30°C. The pathogen was inhibited by 5, 10, and 30 μg each of Aureomycin (chlortetracycline), Chloromycetin (chloramphenicol), Terramycin (oxytetracycline), and tetracycline (Achromycin). The philodendron pathogen was not inhibited at 2, 10, and 100 μg of either dihydrostreptomycin or streptomycin. Erythromycin inhibited the philodendron organism at 5 and 10 μg, but not at 2 μg, whereas penicillin and polymyxin (Aerosporin) inhibited growth at 2, 5, and 10 USP units.

Cross-inoculation studies.—The pathogen induced a soft rot of potato tubers, onions, celery, cabbage, and carrots after 18 hours when artificially inoculated and placed in a moist chamber at 30°C. Unrooted chrysanthemum cuttings inoculated with the philodendron organism were blighted in a manner similar to those inoculated with *Erwinia chrysanthemi* in the same test.

No infections resulted when leaves of *P. selloum* were swabbed lightly with Carborundum before being sprayed with 24-hour broth cultures of either *Erwinia atroseptica* (van Hall) Jennison or *E. carotovora* (Jones) Holland. On a few of the leaves inoculated in a similar manner with *E. chrysanthemi*, however, small pinpoint lesions developed in 48 hours. In repeated tests, chrysanthemum blight organism produced significantly fewer lesions, and disease development was slower, than with the philodendron pathogen (Fig. 1-D). *E. chrysanthemi* and the philodendron organism were passed serially through *P. selloum* 6 times to determine any change in virulence. Injured philodendron leaves were completely rotted when inoculated with the fifth and sixth reisolants. Initial symptoms, however, developed 12–24 hours later with *E. chrysanthemi* than with the philodendron organism. When noninjured philodendron leaves were atomized with 24-hour broth cultures of the latter reisolants, abundant pin-point lesions occurred with the philodendron organism in 24 hours and few to no lesions with *E. chrysanthemi*. *E. chrysanthemi* did not infect uninjured seedlings of *P. selloum* or *P. cannaefolium* Mart. sprayed with a 24-hour broth culture. *E. chrysanthemi* and *E. carotovora* f. sp. *parthenii* Starr infected *P. selloum* seedlings, however, when the leaves were injured with Carborundum before inoculation. Neither of these organisms infected *P. cannaefolium*, whereas the philodendron bacterium proved pathogenic. *Erwinia*

TABLE 1. Major differences between *Erwinia chrysanthemi* and the philodendron pathogen in certain cultural and biochemical reactions, and sensitivity to antibiotics

Media or tests	*E. chrysanthemi*	Philodendron pathogen
Inulin	utilized	not utilized
Indole	negative or trace	strongly positive
NaCl 5%	no growth	good growth
Fermi's; Clara's; Koser's; Ayers'	media turbid but later clears	media turbid & remains cloudy
EMB	violet colonies	violet colonies with metallic sheen
Lactose	weak acid & gas in 2 weeks	weak acid & gas in 1 week
H_2S (tryptone)	negative or trace	strongly positive
Growth at 6°C	positive	negative
Growth at 43°C	positive	negative
Sensitivity to:		
Dihydrostreptomycin	very sensitive	resistant
Streptomycin	very sensitive	resistant
Erythromycin	slightly sensitive	sensitive
Penicillin	slightly sensitive	very sensitive

chrysanthemi did not infect either injured or uninjured leaves of *P. laciniosum*, *P. wendlandii*, or *P. "hastatum"* sprayed with a 24-hour-broth culture. The philodendron pathogen was pathogenic to both injured and uninjured leaves of *P. laciniosum* and *P. "hastatum"* inoculated in a similar manner. In addition, the philodendron pathogen infected *P. wendlandii* only when the leaves were injured with Carborundum before inoculation. Thus, it was shown that *Erwinia chrysanthemi* would not infect certain *Philodendron* spp. that were only moderately susceptible to the philodendron pathogen. It would not infect any of the philodendrons tested unless the leaf surface was injured before inoculation.

Taxonomy of the pathogen.—The morphological, cultural, and biochemical characteristics of the philodendron bacterium place it in the genus *Erwinia* (1). It can initiate a rapid breakdown of philodendron leaves, several fleshy vegetables, and certain pectic substances. Thus, it apparently fits well into the soft rot group of organisms—the *Pectobacterium* group, according to the Waldee classification (15).

In a comparative study, the philodendron bacterium was more closely related to *E. chrysanthemi* than the other species studied. Miller (13) recognized these similarities, but did not describe the pathogen. McFadden, working in cooperation with Miller (11), considered calling the philodendron bacterium a new species, based on the differences reported in Table 1. Since the time when this manuscript was first submitted for publication, 2 papers were published by Graham and Dowson (6, 7), who named the philodendron bacterium, along with *E. chrysanthemi* and others, *Pectobacterium carotovora* var. *chrysanthemi*. Their studies were based on 1 culture obtained from the senior author of the present paper before the pathogen was described. Hellmers (8) reclassified the chrysanthemum blight organism as *Pectobacterium parthenii* var. *chrysanthemi* based on its similarities to *E. carotovora* f. sp. *parthenii*. Burkholder (3) has not adopted the genus *Pectobacterium*, and cites several inconsistencies in following this classification. The present study further supports the conclusions of some workers (2, 3, 10) that *E. chrysanthemi* is a valid species. Even though certain differences exist between the chrysanthemum blight pathogen and the philodendron bacterium, apparently the latter is a strain of *Erwinia chrysanthemi*.

TABLE 2. Control of bacterial leaf rot of philodendron with Agri-mycin 100

Treatment and active concentration	Spray intervals (days)	No. of infected leaves at end of 10 days	20 days	30 days
Agri-mycin 100-200 ppm	4	5	7	7
Agri-mycin 100-200 ppm	8	13	20	23
Agri-mycin 100-400 ppm	8	7	15	16
Control		43	80	128

CONTROL OF THE DISEASE.—Various commonly used fungicides, including Copper A, zineb, captan, and ferbam, have been tested for control of this disease, but none were generally effective. In addition, 3 formulations containing streptomycin were tested against the disease, as both protective and eradicant sprays. The materials included were Agri-mycin 100 (15.0% streptomycin, 1.5% oxytetracycline), Phytomycin (20% streptomycin nitrate), and Agri-Strep (37% streptomycin sulfate). To test possible phytotoxicity of these materials, plants of several *Philodendron* spp. were sprayed 4 times at 5-day intervals with concentrations of 100-800 ppm active material. No phytotoxicity resulted from concentrations of 600 ppm or below, but slight yellowing of the lower foliage resulted from 800 ppm.

To test the effectiveness of each material as protective and eradicant sprays, 5 plants of a philodendron hybrid (*P. wendlandii* × *P. laciniosum*), uniform in age and size, were used for each test. All treatments were repeated at least once. Plants were inoculated by dusting the leaf surface lightly with Carborundum and swabbing them with a cheesecloth pad soaked in a 24-hour nutrient broth culture of the pathogen. All inoculated plants were placed in a moist chamber for 24 hours, and maintained on a greenhouse bench. Visible water-soaked lesions appeared on the control plants 12–24 hours after inoculation.

In general, the materials containing streptomycin were more effective in reducing disease incidence when applied before inoculation than after. Agri-mycin 100 at 200 ppm active ingredient, applied 48 hours before inoculation, completely prevented symptom expression. Phytomycin at 400 ppm active ingredient was almost equally effective. Agri-mycin 100 at 400 ppm active ingredient, repeated at 4-day intervals, gave effective control (Table 2). When applied as a soil drench, the materials were not absorbed and translocated in amounts sufficient to prevent infection or stop disease development.

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BIOASSAY OF TOBACCO RINGSPOT VIRUS IN CASSIA OCCIDENTALIS

D. A. Roberts and M. K. Corbett

Plant Pathology Department, University of Florida, Gainesville.

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SUMMARY

Progressively fewer local lesions formed in leaflets of *Cassia occidentalis* inoculated with increasingly dilute suspensions of tobacco ringspot virus. In 19 tests, numbers of lesions induced in opposite leaflets by inocula differing in viral content by 10, 15, or 20% usually differed significantly. When 2 identical inocula, each at 2 dilutions, were compared in each of 4 potency assays, the differences between actual and measured potencies were 3.1-13.2%. These data show that *C. occidentalis* is a satisfactory local-lesion host for the determination of relative differences in tobacco ringspot activity.

Although several local-lesion hosts of tobacco ringspot virus (RSV) (*Annulus tabaci* Holmes) might be suitable for quantitative work, most bioassays of RSV have been made in cowpea (*Vigna sinensis* (Torner) Savi). *Cassia occidentalis* L. (coffee senna) might be used for this purpose, since localized solid necrotic spots form in leaves inoculated mechanically with RSV (1).

When grown in a 4-in. pot of composted soil, 1 plant of *C. occidentalis* produces as many as 60 pairs of opposite leaflets suitable for inoculation. Thus, satisfactory comparisons of the relative infectivity of 2 inocula can be made in a single plant. *C. occidentalis* has already been used successfully for the bioassay of Cymbidium mosaic virus (2) and potato virus X (3). In work yet to be published, it has proved useful in the correlation of infectivity with characteristic particles observed in electron micrographs of purified preparations of RSV. This paper reports on the suitability of *C. occidentalis* as a bioassay host for RSV.

Materials and Methods.—The virus used in all experiments had been maintained by serial passages through Turkish tobacco (*Nicotiana tabacum* L.). Symptoms induced in tobacco were indistinguishable from those reported (7) as occurring in tobacco plants inoculated with the type strain of RSV. Moreover, recovered leaves showed ringspot symptoms when inoculated with tomato ringspot virus, but no symptoms developed in recovered leaves that were inoculated with RSV.

Locally selected seed of *C. occidentalis* was sown in vermiculite. Seedlings were transplanted singly to steam-sterilized potting soil in 4-in. clay pots. One day before inoculation, plants with 6–8 fully expanded compound leaves were trimmed so that 15–18 opposite leaflets remained on each plant. The Ramshorn Wilt-

Resistant variety of black-eyed cowpeas was used in some tests. Seed was sown in steam-sterilized soil in 4-in. clay pots; 2 plants were allowed to grow in each pot, and primary leaves were inoculated when they had expanded. All leaves were dusted with 500-mesh Carborundum just before inoculation.

The source of inoculum was either inoculated or systemically infected tobacco leaves, harvested 7–14 days after inoculation with RSV. Inoculum was prepared by triturating infected leaves with a mortar and pestle or by grinding with a food chopper. Juice was strained through 2 layers of cheesecloth. All dilutions were made in 0.01 *M* neutral phosphate buffer. The gauze-pad method of mechanical inoculation was used. Each *Cassia* leaflet and each cowpea leaf were rubbed 4 and 6 times, respectively. Inoculated leaflets or leaves usually were rinsed immediately with tap water. In some experiments, however, leaflets of *Cassia* were not rinsed for as long as 10 minutes after inoculation; time of rinsing did not noticeably influence lesion counts.

Dilution Curve and Dilution End Point.—In 1 experiment, RSV in crude juice and at dilutions of 10^{-1}, 10^{-2}, and 10^{-3} was rubbed on leaflets of 12 *Cassia* plants. Each inoculum was replicated 24 times and appeared in each leaf position an equal number of times. Inocula were balanced with respect to left and right leaflet positions and time of rinsing.

Average lesion counts/leaflet were as follows: undiluted, 164; 10^{-1}, 113; 10^{-2}, 28; and 10^{-3}, 5. When lesion counts were plotted on semilogarithmic paper, the slope of the dilution curve was approximately −0.7 in the straight-line portion between points plotted for dilutions of 10^{-1} and 10^{-3}. In 2 tests in which 4 and 5 plants were used, slopes of dilution curves obtained were approximately −0.9. Inocula diluted to 10^{-1} induced 6–24 lesions/leaflet. Results comparable to those reported above and to those reported by Price (4) were obtained in 1 experiment with cowpea as the test plant. Average numbers of lesions/leaf induced by inocula not diluted, and diluted to 10^{-1}, 10^{-2}, and 10^{-3}, respectively, were 253, 153, 94, and 17.

Cassia leaflets and cowpea leaves were rubbed with RSV inocula at dilutions of 10^{-3}, 10^{-4}, and 10^{-5}. Each inoculum was applied to 8 leaves of cowpea and to 24 leaflets of *Cassia*. Only the most concentrated inoculum was infectious in *Cassia*, whereas all 3 were infectious in cowpea. Only 5 lesions were induced in 8 cowpea leaves by the most dilute inoculum.

Although cowpea was more sensitive than *Cassia* to very dilute inocula of RSV, *Cassia* was as reliable as cowpea in detecting large differences in concentrations of active virus in different inocula up to dilutions of 10^{-3}.

Sensitivity of Cassia to Small Differences in RSV Concentrations.—In each of 19 experiments, a "standard" inoculum, usually at a dilution of $10^{-1.5}$, was compared with a "test" inoculum that was either somewhat more concentrated or more dilute. Each comparison was made in 15–18 opposite leaflets of 1 *Cassia* plant. Differences in lesion counts were usually statistically significant by Student's "t" test, when the 2 inocula differed in concentration by 15 or 20% (Table 1), and were occasionally significant when inocula differed by only 10%. In 13 other tests, inocula of the same concentration were compared in 15–20 opposite leaflets of *Cassia*. In 11 of these tests, no statistically significant differences occurred in lesion counts from any 2 identical inocula compared in opposite leaflets. In the 2 tests in which differences in lesion counts were significant, inocula were either crude or clarified juice. Lesion counts were such that if their numbers were plotted against dilution of inoculum, the points would fall outside the straight-line part of the dilution curve.

Table 1. Numbers of local lesions induced in leaflets of *Cassia occidentalis* by different inocula of tobacco ringspot virus

Viral content of test inoculum as % of standard	No. lesions/leaflet[a]	
	Standard[b]	Test inoculum
80	50	48ns
80	58	24**
80	18	11**
85	103	51**
85	67	40**
85	103	46**
85	55	22**
90	37	15**
90	74	39*
110	26	30ns
110	25	27ns
115	40	75*
115	109	133*
115	95	120*
115	132	165**
120	7	14**
120	16	17ns
120	14	36**
120	19	34*

[a] Each inoculum was compared with another in opposite leaflets on 1 plant, and each figure is the average for 15-18 leaflets.

[b] Crude juice diluted to $10^{-1.5}$ in 0.01 *M* neutral phosphate buffer, except in the last 2 experiments, in which the standards were at dilutions of $1.1 \times 10^{-1.5}$.

Results of these tests indicate that *C. occidentalis* is sensitive to small differences in concentration of active RSV, at least between dilutions of 10^{-1} and 10^{-2} in 0.01 *M* neutral phosphate buffer. The ability of *Cassia* to differentiate between inocula differing in RSV concentration by no more than 15% indicates that it is as satisfactory as other plant virus bioassay hosts (5).

Two-Dilution Potency Assays.—In 4 experiments, a "standard" and an "unknown" inoculum were compared in 2-dilution potency assays. Four inocula were prepared, 2 at a dilution of 10^{-1} and 2 at $10^{-1.5}$. One from each group was designated as the "standard," the other as the "unknown." In 1 experiment, each inoculum was compared with all others an equal number of times in opposite leaflets of 12 *Cassia* plants, each of which had 16 pairs of leaflets. Each

inoculum was rubbed on a total of 96 leaflets. In 3 other experiments, identical "standard" and "unknown" dilutions of RSV were compared, each at dilutions of 10^{-1} and $10^{-1.5}$. In 2 of these experiments, the "standard" of a given dilution was compared with the "unknown" of the same dilution in opposite leaves of 2 plants, each of which had 18 pairs of leaflets. In the other test, each of the 6 possible 2-inoculum combinations appeared in opposite leaflets of the same *Cassia* plant; 6 plants were used in this test.

Potencies of the "unknowns" calculated from lesion counts by the formula of Sherwood (6) and expressed as percentage potencies of the "standards," were 96.7, 111.8, 103.1, and 86.8, respectively, in the 4 tests. There results further support the hypothesis that *C. occidentalis* is suitable for the bioassay of RSV. The magnitude of error in potency assays was of the order of that reported for other plant virus assays (5).

DISCUSSION.—Although *C. occidentalis* grows more slowly than the more commonly used local-lesion host, cowpea, 15–60 opposite-leaflet comparisons can be made in a single plant; this minimizes the bioassay error due to plant variation. Further, RSV apparently does not invade *Cassia* systemically, as it does cowpea. Localized lesions that appear in *Cassia* leaflets about 3 days after inoculation are discrete and easily counted. Spots of noninfectious origin have not been observed in leaflets of *Cassia*, whereas purple-brown spots of a noninfectious nature sometimes develop in leaves of cowpea. For these reasons, *C. occidentalis* may often be more satisfactory than cowpea for the bioassay of RSV. Certainly, it can be used with confidence for comparing viral activities of 2 inocula.

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TOBACCO RINGSPOT VIRUS IN SWEET POTATO AND ITS HOST RANGE IN NICOTIANA

E. M. Hildebrand and A. E. Kehr

Plant Industry Station, Beltsville, Maryland.
Respectively, Pathologist and Horticulturist, Crops Research Division, ARS, U.S.D.A., Maryland.
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SUMMARY

The tobacco ringspot virus unmasks the sweet potato internal cork virus in Scarlett O'Hara morning glory. By cross-protection and microprecipitin tests, 2 viruses isolated from foliage lesions of sweet potatoes were identified as strains of tobacco ringspot virus. Alone, neither isolate of the virus produced detectable symptoms on sweet potatoes or on the Scarlett O'Hara morning glory indicator plant. A mixture of internal cork and tobacco ringspot viruses produced typical symptoms of internal cork on sweet potato foliage. In the indicator plant, however, the usual symptoms of the internal cork complex were drastically altered when the tobacco ringspot virus also was present. Instead of the usual shock symptoms followed by masking, the indicator plant developed a chronic mottling and dwarfing of both the foliage and flowers. This synergism between tobacco ringspot virus and the internal cork virus complex in morning glory apparently does not occur in sweet potato. Twenty-five of 102 *Nicotiana* spp. and alloploids tested (not previously reported susceptible) were infected with the sweet potato strain of tobacco ringspot virus.

INTRODUCTION.—Internal cork, a virus disease of sweet potato (12), is commonly transmitted in nature by aphids (7), as are leaf-spot viruses (4) constantly associated with it. Internal cork is the most important disease of sweet potatoes in the United States when the crop is stored, because the market quality of the stored crop may be greatly reduced by the dark-colored, corky lesions in infected roots (6, 11, 12). The effects on yields are hard to estimate (9).

For studying the problems of sweet potato viruses (1), Scarlett O'Hara morning glory has been found of considerable importance because of its excellence as an indexing host for the known sweet potato viruses.

This paper reports finding tobacco ringspot virus masked in sweet potatoes affected with the internal cork syndrome, and its host range in *Nicotiana*.

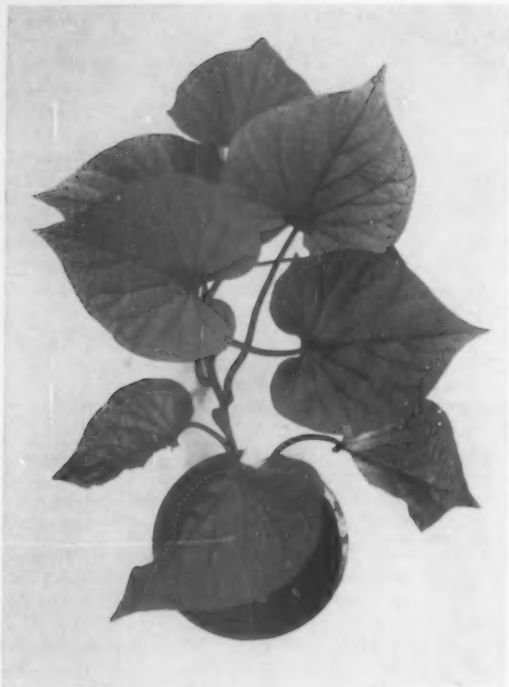


Fig. 1. Acadian sweet potato plant no. 2 showing the largely masked condition of the 3 bottom leaves, which had reacted positively for internal cork virus strain 2 one month earlier.

Indexing for virus.—Two strains have been consistently recovered by inoculation of Scarlett O'Hara morning glory from the foliage and roots of cork-infected sweet potato plants: no. 1, with an incubation period of 5–8 days produces dwarfing, rugosity, vein-clearing, and chlorosis followed by masking; no. 2, with an incubation period of 8–12 days produces moderate to severe vein-banding followed by masking (3). Number 2, originally considered a strain of internal cork, was subsequently found a specific entity, a leaf-spot or ringspot virus (4). The term "strain" is used here for convenience, in the knowledge that more than 1 strain or virus may be involved.

In 1958, plants of Acadian sweet potato, showing a mild chlorotic spotting characteristic of the internal cork virus complex, yielded in addition, a different ringspot virus (5). These plants were produced from roots received in November 1957 from Indiana where tobacco ringspot virus is prevalent in soybeans.

The usual isolation procedure was followed: 5 plants with foliage symptoms typical of internal cork complex were taken from each bedded root after the "flush of growth" period and used for the "squeeze-rub" or rapid manual inoculation on Scarlett O'Hara morning glory (2). This was about 1 month after the Acadian variety with mild chlorotic spotting was potted.

Fig. 1 shows sweet potato plant no. 2 one month after indexing, with the remains of the 3 indexed leaves. The usual leaf-spot symptoms of internal cork are largely masked as the leaves grow older and at no time gave outward evidence of the presence of tobacco ringspot virus. The virus symptoms followed an unusual pattern in the Scarlett O'Hara indicator. The first reaction indicated the presence of strain 1 of internal cork syndrome, but after the usual shock symptoms followed by masking, there was a chronic systemic mottle. The new culture was designated isolate 2 of the internal cork complex.

During 1958, another new culture, designated isolate 3, was recovered from leaf-spot lesions on one 1957 source of Triumph sweet potato in Beltsville. It produced a similar systemic mottle on Scarlett O'Hara indicator. Further attempts to find tobacco ringspot virus in this planting were unsuccessful. Isolate 3 was identified as a virus mixture containing tobacco ringspot virus in the same manner and at the same time as isolate 2.

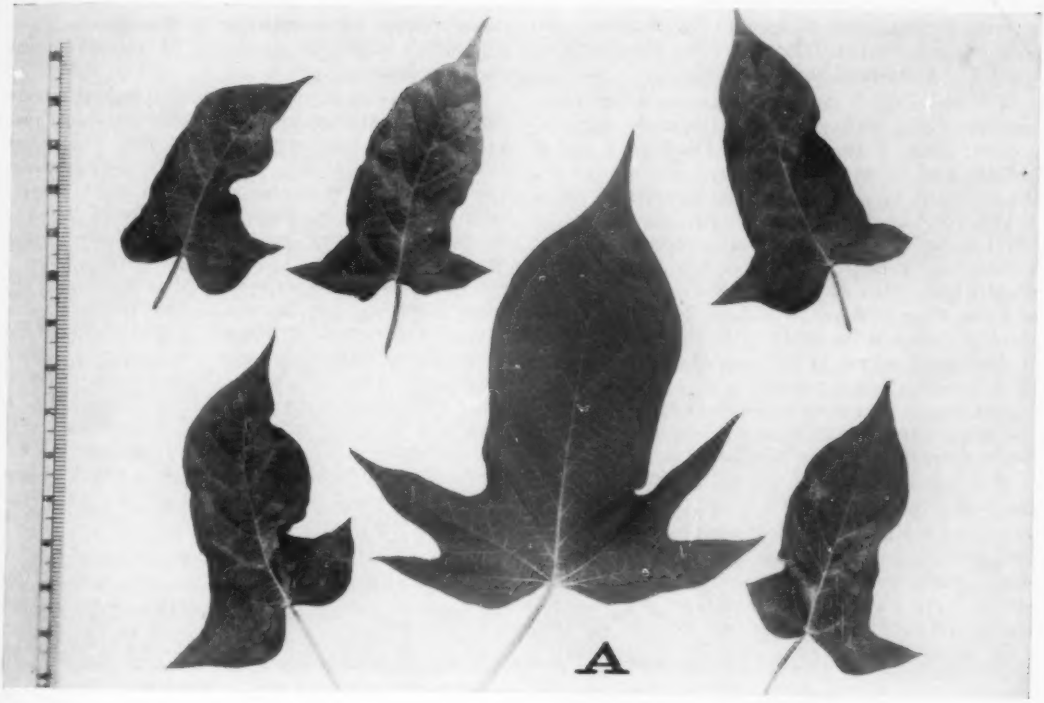
All indicator plants inoculated with isolates 2 and 3 showed shock symptoms on the first-formed foliage. Above this point of demarcation, however, mottle symptoms were noted caused by the associated tobacco ringspot virus, whereas indicator plants inoculated only with strain 1 of internal cork virus complex showed complete recovery above the initial "shock" growth.

After the initial shock phase, leaves from plants inoculated with strain 1 were symptomless and about normal size, whereas those from plants inoculated with isolates 2 and 3 were greatly mottled and much reduced in size (Fig. 2-A). Similar dwarfing plus color-breaking differences were found in the corolla (Fig. 2-B). Blossoms from plants inoculated with isolates 2 and 3 were stunted and had red streaks radiating from the center. The corolla of these flowers contained high titers of tobacco ringspot virus.

The tobacco ringspot virus was only isolated from the bottom leaf of sweet potato plants grown from roots in which it was present, but never from leaves above this point, suggesting that this virus apparently does not multiply readily, or does not become systemic readily in sweet potato.

The modified squeeze-rub or manual method of inoculation transmitted the entire complex from Scarlett O'Hara to Scarlett O'Hara morning glory, whereas aphid inoculation did not transmit the tobacco ringspot component. Reisolation of the tobacco ringspot virus component by the manual method from inoculated sweet potato plants failed. The internal cork component, however, was not only recovered but produced internal cork lesions in the roots of all inoculated sweet potato plants. This finding does not support the reports that aphids can transmit ringspot viruses in association with another virus (10, 13, 14).

Thermal inactivation point.—Employing a procedure for labile viruses, Scarlett O'Hara petioles were placed in test tubes with water and heated at various temperatures for 10 minutes. These thermal inactivation studies were done to separate the internal cork virus complex into its components. Of about 50 isolates gathered over



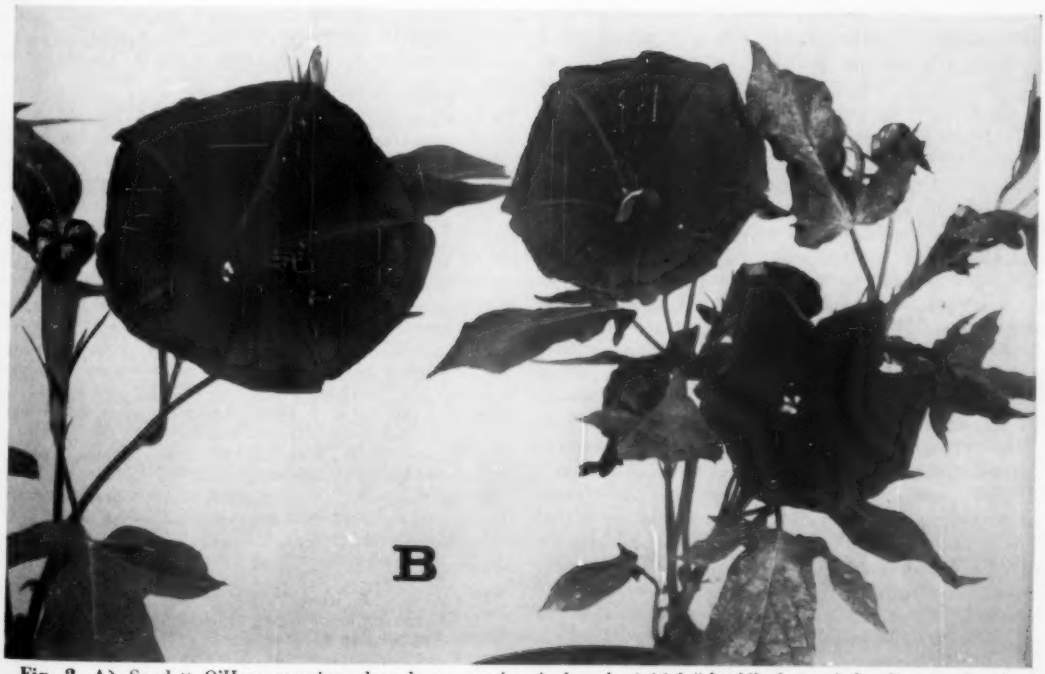


Fig. 2. A) Scarlett O'Hara morning glory leaves produced after the initial "shock" phase of the disease. One leaf (center), from a plant inoculated with strain 1, is symptomless and normal size. The other 5 leaves, mottled and much reduced in size, came from plants inoculated with isolate 2. B) Tobacco ringspot virus causes the scarlet corolla of the morning glory Scarlett O'Hara indicator to become much reduced in size. Also, red streaks radiate out from the center.

a period of years, only 4 appeared significant as possible strains in Scarlett O'Hara morning glory (strains 1 and 2 and isolates 2 and 3).

In March, 1959, 5 thermal inactivation experiments were conducted testing strain 1 (internal cork syndrome), strain 2 (leaf spot), and isolates 2 and 3 (mixtures of strain 1 with tobacco ringspot virus). On the basis of average thermal inactivation points (TIP), the 4 inocula could be placed in 2 groups: 1) 59–61°C (strain 1, isolates 2 and 3); and 2) 56–58°C (strain 2). Thus, strain 2 (leaf-spot virus) has a slightly lower TIP. At present, there is no explanation why the virus mixtures in isolates 2 and 3 behaved essentially the same as strain 1. Results were practically the same, however, in TIP tests of the juice extract from Turkish tobacco, comparing the tobacco ringspot virus component of sweet potato isolates 2 and 3 with the AC 174 strain of tobacco ringspot virus. Respectively, these temperatures were 64°, 64°, and 65°C.

Host range.—Host-range studies of the genus *Nicotiana* were initiated to find indicators for use in separation of the sweet potato virus complex. One hundred and four *Nicotiana* spp. and alloploids were tested during spring and summer by manual inoculation with strain 1 and isolate 2, and sometimes with strain 2 and isolate 3. Many of these were retested the same fall. Strains 1 and 2, the sweet potato viruses, did not infect *Nicotiana*.

Holmes (8) lists 16 *Nicotiana* spp. as susceptible to tobacco ringspot virus, but only 4 of these were duplicated here and found susceptible. Holmes listed 3 suscepts (*N. repanda* Willd., *N. sanderae* Hort., and *N. sylvestris* Spegaz. & Comes), of presumably diploid or 2n chromosome constitution that we found insusceptible in tetraploid or 4n form. This is considered a significant observation.

Positive tobacco ringspot symptoms were produced in 25 of 102 *Nicotiana* spp. or alloploids not included in Holmes' (8) list, using isolates 2 and 3 from morning glory as the source of inoculum. The new suscepts are listed by A. E. Kehr's accession number and name, as follows: 5990 *N. debneyi-cleveiandii*, 5988 *N. langsdorfii-alata*, 59104 *N. longiflora* var. *breviflora* Comes, 5938 *N. bigelovii-debneyi*, 5943 *N. bigelovii-debneyi-tabacum*, 5966 *N. rustica* 'Pearce 4815', 5925 *N. suaveolens-langsdorfii* (doubled), 5914 *N. glauca-langsdorfii*, 5935 *N. attenuata* Torr. ex Wats., 5953 *N. debneyi-plumbaginifolia*, 5960 *N. forgetiana* Hort. ex Hemel., 5964 *N. megalosiphon* Heurck. & Muell. Arg., 5995 *N. excelsior* Black, 5998 *N. goodspeedii* Wheeler, 5999 *N. gossei* Domin., 59106 *N. nesophila* Johnst., 59107 *N. occidentalis* Wheeler, 59108 *N. otophora* Griseb., 59113 *N. rotundifolia* Lindl., 59117 *N. solanifolia* Walpers, 59118 *N. stenocarpa* Wheeler, 59120 *N. tomentosiformis* Goodsp., 59122 *N. velutina* Wheeler, 5937 *N. bigelovii* var. *Wallacei* A. Gray, and 59122 *N. pauciflora* Remy.

Identification of tobacco ringspot virus.—When *Nicotiana* host range studies pointed to the probability that sweet potato isolates 2 and 3 contained a known ringspot virus, cross-protection tests were conducted on Turkish tobacco to determine the identity of the ringspot virus.

Known strains of tobacco ringspot and of tomato ringspot viruses were used for inoculating plants that had recovered from symptoms produced with sweet potato isolates 2 and 3. On the basis of these trials, tomato ringspot virus was eliminated as a suspect when it produced symptoms on the infected tobacco; no further symptoms developed when infected leaves were inoculated with tobacco ringspot virus.

This material was further tested in the Plant Virology Laboratory at Beltsville. The microprecipitin test (15), performed by Howard A. Scott, showed the sweet potato ringspot virus was serologically related to the type strain of tobacco ringspot virus (AC-174, Catalogue and Registry of the Plant Viruses, Ed. 2, American Type Culture Collection, Washington, D.C.; the antiserum was produced by Microbiological Research Foundation in collaboration with The American Phytopathological Society Committee on Virus Type Culture Collections).

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TRANSMISSION OF TOBACCO MOSAIC VIRUS IN TOMATO SEED

R. H. Taylor, R. G. Grogan, and K. A. Kimble

Respectively, Senior Plant Pathologist, Department of Agriculture, Victoria, Australia; Professor of Plant Pathology; and Laboratory Technician IV, University of California, Davis.

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SUMMARY

The location of tobacco mosaic virus (TMV) in tomato seeds from infected plants is in and on the seed coat, and in a small percentage of the endosperms, but not in embryos. Seed-coat virus was eliminated by acid extraction or trisodium phosphate treatment, but not by thorough washing in detergent solutions. Virus in endosperms was not affected by acid or Na_3PO_4 treatments, but was inactivated slowly during storage.

Seedlings grown from infected seed were contaminated, but not infected with virus. Contamination was common on roots, but occurred on cotyledons only when the seed coats were elevated during germination. Embryonic seed transmission of TMV in tomato occurs rarely, if ever; and was not demonstrated in this study. Nevertheless, infection from the virus contaminating the roots and cotyledons may occur during transplanting, and seed-borne virus is an important source of inoculum.

The transmission of tobacco mosaic virus (TMV) in seed from infected tomato plants and the location of TMV in infected or infested tomato seed have been the subjects of numerous investigations. The results, however, have been contradictory, leading to considerable confusion and disagreement on the importance of seed transmission in the etiology of the disease. This report is concerned with: 1) the location of TMV on or in the seed, 2) the amount and the mechanism of seed transmission of TMV in tomato, and 3) the effects of different methods of seed extraction and treatment on the elimination of TMV from tomato seed and their effect on seed transmission.

REVIEW OF LITERATURE.—Several investigators (10, 17, 19, 21) have reported no seed transmission of TMV, in thousands of seedlings grown from seed produced by infected plants. For example, Jones and Burnett (17) reported no seed transmission to 3,367 plants grown from seed from TMV-infected plants. They stated that "these diseases (mosaic and streak) do not appear on the plants until they have been handled by workmen. . ." Wildman (23) reported no knowledge of any confirmed report that TMV is ever transmitted through seeds. Others (2, 7, 8, 9, 11, 14, 15, 20, 22), in contrast, have reported various amounts of seed transmission. Two of the reports (15, 22) indicated that TMV was transmitted in freshly extracted seed, but not in stored seed. Doolittle and Beecher (15) reported that storage of seed reduced the amount of seed transmission and also caused a delay in symptom expression. Berkeley and Madden (7, 8) presented evidence that TMV is present in embryos of seed produced by infected plants. Crowley (13, 14), however, was unable to demonstrate TMV either in endosperms or embryos and suggested that seedlings became accidentally infected by virus from seed coats during germination. Allard (3) wrote: "Although in the writer's experience no evidence of embryonic infection in the seed has ever been obtained, it would not be safe to assert that it never occurs. However, all evidence at hand indicates that it must be of very rare occurrence."

Chamberlain and Fry (11) compared uncleaned, fermented, and acid-extracted seed with respect to virus content (indicated by assay of composite samples on local-lesion hosts) and amount of seed transmission. They reported that TMV was transmitted by uncleaned seed, but not by seed extracted by fermentation or by acid. Virus concentration was highest in the uncleaned seed; was reduced by fermentation; and was nearly, but not completely, eliminated by acid extraction. They concluded that the virus is carried only on the surface of the seed, and suggested that seedlings become infected during or after emergence. On the basis of failure to eliminate all seed-borne virus by detergent treatments, Crowley (14) disagreed with Chamberlain and Fry (11). He concluded that the virus was not merely a superficial contaminant, but also infected the seed coat; and therefore would not be eliminated by acid extraction.

Several other reports have been concerned with the effect of different times and methods of storage (15, 18, 22) and seed treatments (2, 11, 21) on the reduction or elimination of TMV in tomato seed. John and Sova (18) reported no reduction in the amount of TMV in 3-year-old seed held in "common" storage, but complete loss of infective virus in small seed samples stored 7 months in paper envelopes in the laboratory. They also reported that trisodium phosphate treatment inactivated the virus in seed. Nitzany (21) and Alexander (2), in contrast, reported that trisodium phosphate treatment of seed only reduced the amount of virus

without eliminating it. Alexander (2) found no virus in seed from mature infected fruit extracted with HCl. Nitzany (21), in agreement with Chamberlain and Fry (11), concluded that the virus was not completely eliminated by acid extraction.

It was not possible to determine by a review of the literature whether the conflicting reports are due to differences in tomato varieties, as suggested by Milbrath (20), or to differences in methods of investigation.

MATERIALS AND METHODS.—Seeds of the varieties Cal Pac 2, Ace, and Moscow were obtained from TMV-infected plants grown at Davis, Calif. Seed samples of other varieties were obtained from infected plants in a tomato breeding plot at Burnley Gardens, Victoria, Australia. Seed was extracted: 1) by squeezing pulp from fruit on a wire screen and allowing it to dry without further treatment (direct extraction in tables and text hereinafter), 2) after fermentation, or 3) after acid treatment. In the last method, described by Chamberlain and Fry (11), seed and pulp were squeezed into a glass container, and concentrated HCl was added at the rate of 25 ml of acid to the pulp and seed from 5 lb of fruit. The mixture was stirred occasionally during a 3-hour treatment. Then, the seed was screened out, washed with tap water, and dried. The trisodium phosphate treatment, similar to that described by John and Sova (18), consisted of soaking seed for 1 hour in 10% solution of Na_3PO_4, followed by dialysis against tap water overnight. In the detergent treatments, seed was shaken for 12 hours in 10% lauryl sulphate solutions, then washed in 5 changes of water, with shaking for 1 hour in each change of water. To test for contaminating (loosely held) virus on seed coats, 20 seeds were washed for 5 minutes in 1 ml of pH 7 phosphate buffer and the washings were inoculated to local-lesion assay hosts.

To test for viable TMV in seeds or seedlings, inoculations were made to *Nicotiana glutinosa* L., *N. tabacum* L. var. *Xanthi* N.C., or *Datura stramonium* L., using either a conventional mortar and pestle or a Plexiglas board drilled with shallow holes to form a composite mortar. Pestles for the Plexiglas board were glass rods with rounded ends. Leaves of test plants were inoculated by rubbing with the pestles or glass rods used to grind the inoculum. During inoculation, leaves were supported by a wooden pot label, which was discarded after use. Phosphate buffer (0.1 *M*, pH 7) and 600 Carborundum were used in all inoculations; the inoculum was not washed off. Depending on the supply of test plants, whole leaves, half leaves, or interveinal sections of leaves were inoculated. Although all lesions were counted, counts are given only when particularly significant; since, usually, the presence or absence, rather than relative titer, of virus was being determined. Infected tomato seedlings usually expressed clearly evident symptoms. Nevertheless, in much of the work, seedlings were indexed on local-lesion hosts to detect possible masking of symptoms.

Throughout this work, every effort was made to eliminate contamination: all apparatus was sterilized before use; scalpels, tweezers, and slides were flamed after each dissection; plants were widely spaced on the greenhouse bench and were not handled. Other methods are described along with results of the particular experiment.

RESULTS.—1) *Effect of method of extraction, treatment, or storage on TMV content of whole seeds.*—The work of Chamberlain and Fry (11) was repeated, with results in close agreement: that fermentation followed by washing reduced virus more than direct extraction and that acid treatment eliminated most, but not all, TMV from the seed.

Similarly, a trace of virus could always be detected in a bulk sample of 100 seeds treated with Na_3PO_4, indicating that this method was not completely effective in eliminating TMV. This agrees with the findings of Alexander (2) and Nitzany (21). Prolonged washing of directly extracted seed with 10% detergent solution, followed by 5 one-hour rinses in water, failed to eliminate TMV, which agrees with Crowley (14). When detergent-treated seed was shaken for 5 minutes in 0.1 *M* pH 7 phosphate buffer, however, no virus could be detected by inoculating local-lesion hosts with the washings. In contrast, washings from directly extracted fresh or 6-month-old seed were highly infectious. When 18-month-old seed stored in paper bags in the laboratory was tested similarly, however, no virus was detected in the washings. Some virus, however, was detected in ground extracts of whole seeds of 8 different seed samples. Thus, the virus on surface of seed can be removed by prolonged washing or can become inactivated in storage. Some virus is more deep-seated, however, and is neither removed by washing nor inactivated as quickly in storage.

These results with bulk 100-seed samples indicated that acid extraction, Na_3PO_4 treatments, and storage for several months were only partially effective in eliminating TMV from seed. The results did not indicate whether a smaller amount of TMV was present in all formerly infected seed or whether the percentage of infected seed was reduced by the treatments. This was determined by assays on local-lesion hosts of inoculum prepared from individual seeds. The results (Table 1) show that a high percentage of fresh directly extracted seed had TMV and that the percentage was reduced somewhat by 12–14 months of storage. Both acid extraction and Na_3PO_4 treatment markedly reduced the percentage of infected seed. Both treatments left, in freshly extracted seed, about 2–3% infected seed, which was usually reduced in 12–14 months of storage to about 1% or less. The small percentage of infected seed from both acid-extracted and Na_3PO_4-treated samples had high virus titers, indicating that the treatments had not greatly reduced the virus content of these seeds. These results explain why Chamberlain and Fry (9), Alexander (1), and Nitzany (19) failed to eliminate all TMV from seeds similarly treated.

2) *Effect of method of extraction, treatment, and storage on TMV content of different portions of dis-*

TABLE 1. Results of inoculations from single whole seeds to determine the presence of TMV in seed subjected to different treatments and times of storage

Variety	Treatment	Age of seed (months)	Number indexed	
			Positive	Total
Grosse Lisse	Directly extracted	0	185	200
Grosse Lisse × Belle 95	" "	0	85	100
Grosse Lisse × Belle 95	" "	14	52	100
Cal Pac #2	" "	12	21	66
Ace	" "	12	60	66
Moscow	" "	12	101	132
Cal Pac #2	Acid extracted	12	4	366
Grosse Lisse × Belle 95	" "	0	1	100
Grosse Lisse	" "	0	15	400
Grosse Lisse × Belle 95	Trisodium phosphate	0	3	100
Grosse Lisse × Belle 95	" "	14	2	500
Grosse Lisse	" "	0	3	200
Grosse Lisse	" "	12	1	400

sected seed.—To determine more exactly the location of seed-borne TMV, seed from infected plants that had been extracted, treated, or stored in various ways was dissected into seed coats, endosperms, and embryos. Each portion was ground separately and assayed for virus. Seeds to be dissected were soaked overnight in water in petri dishes, and teased apart with a sharp, pointed scalpel under a dissecting microscope. The seed coat was easily removed without damaging the endosperm, which is covered by a thin, tough cuticle. This layer was usually removed and included with the seed-coat portion, but including it with the endosperm did not appear to alter the results. Removing the embryos without excessive injury from within the endosperm was somewhat more difficult, because the cotyledons are wrapped around a central column of endosperm tissue. Even so, by making small cuts in the endosperm tissue, the embryos could be completely freed and removed intact. To avoid spurious results caused by possible surface contamination, embryos and endosperm were washed in 10% lauryl sulfate solution and rinsed in water before being ground for TMV assay. Similar washing and rinsing of known infected leaf and root tissue showed that this treatment had little or no effect on TMV in infected tissues.

The results (Table 2) showed TMV in a high percentage of seed coats from directly extracted seed. Only a small percentage of the endosperms and none of the embryos were infected. Treatment of directly extracted seed with detergent reduced the percentage of seed coats with TMV, but did not eliminate it. In contrast, both Na_3PO_4 treatment and acid extraction practically eliminated TMV in the seed coats. In fact, only 1 seed coat in 470 gave a few local lesions when assayed, and it came from a seed in which over 200 lesions developed when the endosperm was assayed. It is probable, therefore, that the seed coat had become contaminated with virus from the endosperm tissue. The percentage of infected endosperms in these experiments is in close agreement with the number of whole seed infected following acid extraction or Na_3PO_4 treatment (Table 1).

3) *Effect of age of seedlings and plant part inoculated on susceptibility to TMV.*—Freshly extracted embryos were placed in petri dishes on moist filter paper, dusted with Carborundum, and inoculated by brushing TMV inoculum from infected tomato leaves on the whole embryo surface with a short-bristled camel's hair brush. The inoculated embryos were transplanted to steam-sterilized soil. After 10 days, each seedling was assayed for TMV infection on a local-lesion host by sampling from the first true leaf. None of 30 seedlings was infected, indicating that embryos are highly refractory or possibly immune to infection. Therefore, it was of interest to determine when developing seedlings become susceptible. That was the purpose of the following experiment: acid-extracted seeds were germinated on moist filter paper. Radicles, hypocotyls, and cotyledons were inoculated as the respective seedling parts became exposed during germination. The seedlings were transplanted and assayed for TMV infection 10 days after inoculation by sampling the first true leaf, as above. The results (Table 3) indicate that radicles are more difficult to infect than other plant parts, or that possibly the virus moved very slowly from infected roots to above-ground parts of the plant, as shown by Fulton (16). Inoculation of hypocotyls and cotyledons, however, also failed to infect all of the seedlings until 7–9 days after planting, indicating that very young seedlings are refractory to infection, but later become more susceptible.

4) *The location of TMV on seedlings grown from directly extracted seed.*—Preliminary experiments showed that TMV was frequently associated with unwashed roots of seedlings grown from directly extracted TMV-infected seed, but not with unwashed above-ground parts or with roots that had been washed with detergent solution and rinsed in water. The latter observation suggested that the roots were not infected with virus, because similar washing and rinsing of roots from known infected plants did not reduce the titer of virus in inoculum preparations made from them. The reason for the scarcity of virus on the

TABLE 2. Effect of different methods of seed extraction and treatment on recovery of TMV from different portions of dissected tomato seed

Variety	Methods of seed extraction and treatment	Age of seed (months)	Seed part indexed	No. positive/no. indexed
Master Marglobe	Direct extraction	0	Seed coat	18/20
			Endosperm	2/20
			Embryo	0/20
Grosse Lisse	Direct extraction	0	Seed coat	18/20
Grosse Lisse	Direct extraction + washed in detergent	0	Seed coat	27/40
			Endosperm + embryo	1/40
Grosse Lisse × Belle 95	Direct extraction + trisodium phosphate	0	Seed coat	0/30
			Endosperm + embryo	1/30
Grosse Lisse × Belle 95	Acid extraction	4	Seed coat	0/240
			Endosperm	1/240
			Embryo	0/240
Grosse Lisse	Acid extraction + trisodium phosphate	0	Seed coat	1/200[a]
			Endosperm	5/200
			Embryo	0/200

[a] Probably due to accidental contamination from corresponding endosperm which was heavily infected.

above-ground plant parts was not immediately apparent. It was observed, however, that the seed coat, though usually left underground when the seedling emerges, is occasionally elevated by hanging on the tips of the cotyledons. Assays for TMV were made from seedlings that had elevated the seed coat, and from others whose seed coats were left below-ground. The results (Table 4) indicate that TMV was associated with both roots and above-ground plant parts when the seed coat was elevated during germination, but was associated only with roots when the seed coat remained below-ground. The virus also was eliminated from both above- and below-ground plant parts by washing in detergent solution, thus confirming previous indications that the seedlings were contaminated but not infected.

5) *The mode of seed transmission of TMV.*—TMV is so infectious and so easily transmitted mechanically that the percentage of natural seed transmission must be based on data obtained from direct-seeded plants that have not been handled. In this study, about 2,500 seedlings were produced by direct-seeding of fresh directly extracted seed, heavily contaminated with TMV. Only 2 seedlings of the variety Master Marglobe became infected. Furthermore, symptoms did not develop until the third true leaf had formed, indicating that transmission probably was due not to embryonic infection but to accidental infection that occurred during or at some time after germination.

Chee (12) reported that Southern bean mosaic virus invades a high percentage of immature embryos of bean. It is inactivated or inhibited as the seed matures, thus greatly reducing and usually eliminating seed transmission in mature seed. To determine whether a similar relationship exists with TMV in tomato seed, embryos of 78 seeds from green infected fruit were dissected out, washed in detergent, and assayed separately for TMV. Although virus was readily demonstrable in the fruit tissues, none of the embryos contained virus. Thus, it appears that TMV does not affect embryos at any stage of their development.

Fulton (16) reported that TMV moves very slowly from infected roots into above-ground parts. An experiment was conducted to determine whether radicle infection occurs during germination; and, if so, whether the virus later moves into the tops. Fifty plants were grown by direct-seeding heavily contaminated seed in separate pots kept in complete isolation and not handled for 4 months. The roots and tops from each plant were assayed separately on local-lesion hosts, but none was infected with TMV. These results, as well as the data (Table 4) showing that seedlings grown from seed contaminated with TMV are frequently contaminated but not infected with the virus, suggested that contaminated seedlings might become infected by mechanical abrasions received during transplanting. The results of transplanting experiments (Table 5) confirmed this hypothesis. No infection occurred when seedlings were not transplanted. A high percentage of Grosse Lisse seedlings grown from directly extracted fresh seed, however, became infected when transplanted either from soil or vermiculite. Seedlings transplanted from vermiculite, a medium from which a higher percentage of seed coats was elevated, were

TABLE 3. Effect of age of plants and plant part inoculated on susceptibility of tomato seedlings to TMV

Days after planting	No. indexed positive/no. indexed			
	Part inoculated			Uninoculated control
	Radicle	Hypocotyl	Cotyledons	
2	3/15			0/15
3	4/15	4/14		0/13
4	7/17	6/12	5/12	0/16
5	3/18	14/19	11/15	0/16
6	8/16	7/13	12/13	0/13
7	4/12	11/11	14/17	0/16
9	5/11	11/11	14/14	0/11

TABLE 4. The effect of position of seed coat after germination on the portion of tomato seedlings infested with TMV

Position of seed coat after germination	Plant part indexed				
	Seed coat	Root-hypocotyl		Cotyledons-epicotyl	
	unwashed	Washed[a]	Unwashed	Washed	Unwashed
Elevated	37/75[b]		16/50	0/35	21/75
Not elevated		0/275	126/598		0/408

[a] Shaken in detergent solution, followed by rinsing in water.
[b] Number indexed positive/total.

infected in higher proportions than comparable samples transplanted from soil. This result substantiates the data (Table 4) showing that contamination of cotyledons occurred only when the seed coat was elevated during germination. Likewise, the higher proportion of infected seedlings of Grosse Lisse is probably due to this variety's propensity for frequent elevation of the seed coat, unlike Ace seedlings, whose seed coat usually remains below-ground.

No infection occurred in any of the seedlings grown from 14-month-old seed or seed that had been extracted with acid. This indicates that TMV in endosperm tissue and within the seed coat rarely infects seedlings. Very large numbers of seedlings would have to be transplanted to verify that transmission never occurs; or, if it does, to determine the frequency of transmission from these sources.

DISCUSSION.—The term "seed-borne virus" as used here refers to virus carried in or on any portion of the seed. Virus-host combinations in which the virus infects embryos usually result in consistent seed transmission. If the virus is carried in some other part of the seed, as is TMV in tomato, seed transmission may occur but is inconsistent, since it is dependent on the nature of the virus and other fortuitous circumstances.

Bennett (4, 5) listed a number of virus-host combinations in which the viruses are consistently seed-transmitted and pointed out that the number of non-seed-transmitted viruses is much larger. He hypothesized that seed transmission may depend on the ability of the viruses to enter the embryo sac, either directly or through the pollen tubes, and to infect and survive in the embryos. This type of seed transmission is "embryonic"; and should be clearly distinguished from other, more common, virus-host combinations in which the viruses are usually not seed-transmitted, but may be seed-borne. For example, Bennett and Esau (6) have shown that curly top virus invades all parts of the beet seed except the embryos, and that viable virus is still present in seed coats after germination. Thus, this virus is seed-borne, although extensive tests indicate that it is not seed-transmitted.

It seems likely that the lack of agreement in the literature concerning seed transmission of TMV in tomato is due to the failure of the virus to seed-transmit consistently. The reasons for the inconsistency have not been elucidated satisfactorily. Several reports (1, 11, 13, 14) have indicated that the virus does not infect and survive in tomato embryos. Some (1, 11, 13, 14, 19) have suggested that seed transmission results from fortuitous inoculation of germinating or older seedlings with virus originally present in or on seed coats. Our results agree with and support this interpretation and help to explain why TMV is only occasionally seed-transmitted, even when seed is contaminated heavily. For example, seedlings grown from heavily contaminated seed were themselves contaminated, but were not infected. A portion became infected when transplanted, but the proportion infected was dependent on whether cotyledons or roots were contaminated. This, in turn, depended on whether seed coats were elevated or left below-ground during germination.

Some workers (11, 20, 22) have reported seed transmission of TMV to nontransplanted seedlings. Our results indicate that this happens rarely if ever, in the varieties we tested. It seems likely that it might happen occasionally with a virus as infectious as TMV. We were unable to obtain seed of the Indiana canner variety that Milbrath (20) reported was the only 1 in his experience that seed-transmitted TMV to untransplanted seedlings. It would be interesting to know how

TABLE 5. Effect of method of extraction, aging of seed, and germination medium on amount of infection occurring during transplanting

Variety	Extraction method	Age of seed (months)	No. infected/no. indexed			
			Transplanted from		Control (untransplanted)	
			Soil	Vermiculite	Vermiculite	Sand or soil
Grosse Lisse	direct	0	15/55	16/20	0/56	
Grosse Lisse	direct	1	2/100	21/49	0/52	0/100
Grosse Lisse	direct	14	0/415		0/156	
Grosse Lisse	acid	1	0/890		0/250	
Ace	acid	0	0/233			0/234
Ace	direct	0	3/202	2/100	0/103	0/248

this variety differs from others less prone to seed-transmit TMV. Our results suggest that it might elevate a higher proportion of the seed coats during germination, and possibly that inoculations might occur when the seed coat is pulled off the cotyledons. It also is possible that this might be an instance of embryonic seed transmission of TMV in tomato, but this seems unlikely. Our results indicate that TMV infection of tomato embryos occurs rarely, if ever. This is in agreement with other investigators, except Berkeley and Madden (8), who reported infection of healthy plants by inoculations with crushed embryos from diseased plants. The method used by them for excising embryos was not described. Unless precautions were taken to prevent contamination from other seed parts, the results would be meaningless.

The demonstration in this study that a small percentage of seeds from TMV-infected tomato plants has virus in the endosperms provides an explanation of the small residue of TMV reported in seed after extraction with acid or treatment with trisodium phosphate (2, 11, 21). Our results showed that both treatments eliminate any TMV in or on the seed coat. Neither treatment affects the endosperm virus. It seems likely that both acid and phosphate can permeate the nonliving seed coats, but are incapable of penetrating the living membranes of the endosperm tissue. It also seems likely that germination would have been adversely affected had the materials penetrated to the embryos. That this did not occur was indicated by the fact that increasing the time of acid extraction from 3 to 24 hours did not reduce the percentage of seed germination.

No infection resulted in transplanted seedlings grown from 18-month-old directly extracted or from fresh acid-extracted seed, although assays of the seed showed viable TMV present in seed coats of the former and endosperms of the latter. These data indicate that loosely held TMV on the surface of fresh directly extracted seed is most likely to result in infection of seedlings. Thus, it is likely that extraction by acid or by trisodium phosphate, or simply by washing with detergent—treatments that remove most or all of the superficial virus—would reduce, or in most cases prevent, seed transmission of TMV in tomato. Virus in endosperms and in the seed coat did not cause infection in our tests. Nevertheless, since it was still infectious after germination was complete, the virus cannot be ignored as a potential source of inoculum without exhaustive tests of many more seedlings than was possible in this study.

It is concluded that seed-borne TMV is an extremely important source of inoculum. If control of the disease in tomato is to be assured, this source and other possible sources of the virus must be eliminated.

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HULL ROT DISEASE OF ALMONDS

C. J. Mirocha and E. E. Wilson

Department of Plant Pathology, University of California, Davis.

Respectively, Plant Pathologist, Union Carbide Research Station, Clayton, North Carolina; and Professor of Plant Pathology, University of California, Davis.

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SUMMARY

The pathogenic role of *Rhizopus stolonifer*, *R. circinans*, *R. arrhizus*, *Monilinia fructicola*, and *M. laxa* was demonstrated in hull rot and twig blight of almond. Most frequently associated with the disease were *Rhizopus* spp. and their role was studied further.

The disease is characterized by the rotting of the fruit mesocarp (hull), with subsequent blighting of the adjoining twig and leaves. Both the vascular discoloration of the twig and the necrosis of leaves occur on the side of the stem that has the same vascular connection as the peduncle of the rotted fruit. Isolation and histological data showed that the organism is not present in the tissue of the blighted twig. Consequently, a translocated toxin is postulated as the agent responsible for twig and leaf symptoms. A search for the toxin is the subject of a later paper.

INTRODUCTION.—Hull rot, a disease of almonds present in the Sacramento and San Joaquin valleys of California for many years, was formerly believed caused only by the 2 brown rot fungi [*Monilinia fructicola* (Wint.) Honey and *M. laxa* (Aderh. & Ruhl) Honey]. Recently, however, it became apparent that *Rhizopus* spp. were most often associated with the disease.

This paper describes the symptoms of the disease, its cycle of development, and reports evidence that *Rhizopus* spp. are frequently the causal agents.

MATERIALS AND METHODS.—All isolations were made from host tissue cut from the margins of the diseased area of hull, twig, and peduncle. The diseased hulls were first surface-sterilized with 70% ethyl alcohol (1 minute) and 0.8% NaOCl (2–3 minutes); peduncles and stems were kept for 1 minute in the former and 5 minutes in the latter. Tissues were incubated on potato-dextrose agar (PDA) for 5 days at 12°C and then at room temperature. This sequence allowed slow-growing organisms to make their appearance before they were overrun by fast-growing organisms. Isolations were made over a period of 3 years from material found in the major almond-producing areas of the Sacramento and San Joaquin valleys.

Spores of fungi (water suspension) isolated from affected almond fruits were obtained from single-spore cultures and inoculated into dehiscing fruit by placing 1 ml of the inoculum in the open suture. Some inoculated fruits were left uncovered; others were covered with a polyethylene bag. All controls were located on the same limb as inoculated fruit.

Moisture content of almond fruits during various stages of dehiscence was determined in the following manner: 10 fruits, selected for uniformity of size and suture dehiscence (0.25 mm), were marked and left on the trees. Every second day, the suture opening (width) was measured on each fruit and the average taken. Then, 10 other fruits, of comparable size (on the same tree) and with the same average suture opening, were harvested and the fresh weight determined. The hulls then were dried 72 hours at 105°C and reweighed.

RESULTS.—*Symptoms.*—Three symptomatic expressions are associated with the hull rot disease: 1) rotting of hulls, 2) leaf blighting, and 3) killing of twigs. The last seems most important because it affects production most. Usually, the kernel of the fruit is not affected.

Late in summer, after the almond hulls have begun to dehisce, irregular light-brown necrotic lesions appear on the mesocarp (hull). These later coalesce, and the entire hull becomes wrinkled and shrunken. Usually, *Rhizopus* can be seen sporulating on the inner surface of the hull.

Leaves of the clusters nearest the rotted hull curl up and later become brittle; some develop unilateral necrotic areas in the blades and necrotic veins. The latter is more common on shoots of the current year. The blighted leaves remain attached to the spur (Fig. 1). Brown to black streaks develop in the vascular tissue along the same side of the twig which bore the infected fruit. Spurs and twigs bearing the rotted fruit die later, often including twigs up to 18 in. long.

On a shoot with a rotted fruit at its base, necrosis occurs in those leaves that arise from the same side of the shoot as the diseased fruit (Fig. 5). Moreover, these leaves develop necrotic areas only along 1 side of the midvein. Twigs that bear a number of infected fruit, however, may not show such unilateral symptoms.

Microscopic symptoms.—In diseased twigs, cavities filled with gum form in the region of the cambium (Fig. 2). These gum pockets enlarge into gum ducts between the vascular rays. Gum, staining red, orange, or yellow with phloroglucinol and HCl, was found in some of the mature xylem vessels.

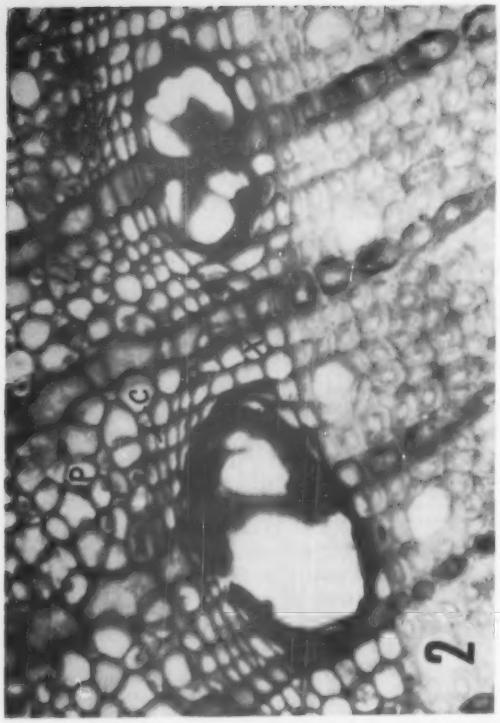

Fig. 1–4. 1) Left, healthy fruit and shoot of Nonpareil; right, hull rot and twig blight on shoot of Nonpareil. Note more than 1 infected fruit on the twig. 2) Cross section of stem showing early stages of gum pocket development at about the time first signs of leaf symptoms are evident. × 360. P, phloem; C, cambium; X, xylem. 3) Transverse section of almond twig affected by hull rot showing gum pockets deep in the xylem. The diseased twig was harvested and sectioned 2 months after initial symptom development. 4) Fruits of Ne Plus Ultra at different stages of dehiscence. Left to right, incipient and 8 days later.

Tissue in which the gum pockets were found was xylem (Fig. 2). The cells were as yet unlignified and were readily stained with fast green. When partly blighted twigs, bearing infected fruit on orchard trees, were marked in August and harvested in October, histological sections again showed gum pockets in the xylem (Fig. 3). They were then discrete islands surrounded by xylem tissue, apparently isolated by the formation of new xylem cells that matured between August and October.

Isolations.—Isolations from rotted hulls attached to blighted stems most often yielded *Rhizopus* spp. Table 1 lists the organisms isolated during 3 years, with their frequency of isolation. *M. laxa* and *M. fructicola* were identified on PDA by methods of Hewitt and Leach (1). The *Rhizopus* spp. were identified by the keys of Zycha (4) and of Kockova-Kratochvilova and Palkoska (2). All other fungi were identified by use of common mycological keys.

Rhizopus spp. and *M. fructicola* were found most often in peduncles. *M. fructicola* was never isolated from the adjoining stem-tissue, whereas *Rhizopus* spp. were isolated in 11.9% of the trials. Such isolations from twigs were considered cases of accidental contamination because numerous histologic sections stained with safranin, lacto fuchsin, or phloxine B never revealed any fungus mycelium in blighted twigs. Moreover, special care was taken to detect any mycelium in the gum pockets, in both their early and late stages of development. All were negative.

Rhizopus spp. encountered were *R. stolonifer* (Ehr. ex Fr.) Lind. 1913, *R. circinans* v. Tilgh. 1876, and *R. arrhizus* Fischer 1892.

Pathogenicity of isolates.—On July 26, 1957, dehiscing fruits of the almond variety Nonpareil were inoculated—100 fruits with *R. stolonifer* and 154 with *R. circinans*. A rapid rotting of fruit and twig blight ensued. In *R. stolonifer*, 48% of the hulls were rotted and 17% of the twigs developed leaf blight. In *R. circinans*, 19% of the hulls were rotted and 2.5% of the twigs developed leaf blight. The controls in both cases remained normal.

R. stolonifer also was inoculated on 50 dehiscing fruits of Ne Plus Ultra on August 20, 1957. This variety dehisces later than Nonpareil. Of these fruits, 56% of the hulls were rotted; 28% of the total inoculations resulted in leaf blighting. Symptoms were evident 10 days after inoculation. *R. stolonifer* was readily reisolated from hulls and peduncles.

Similar inoculations with *R. arrhizus* also demonstrated its ability to produce the hull rot disease.

Rhizopus spp. also were capable of rotting immature, green fruit inoculated through wounds on orchard trees in May. Blighting also developed in leaf clusters nearest the rotted fruit.

In 2 separate experiments, both *M. laxa* and *M. fructicola* were inoculated on 31 dehiscing fruits of Nonpareil and 50 fruits of Ne Plus Ultra. Symptoms developed in 6 days. Fifty-five % of the fruits were rotted by *M. laxa;* 22% of the twigs developed leaf symptoms. *M. fructicola* rotted 62% of the fruits, and produced leaf blighting and twig symptoms in 17% of the cases. As in natural infections, the embryo of the fruit was not affected.

Isolates of *Botrytis* sp., *Stemphylium* sp., and *Alternaria* sp. also were tested for pathogenicity. Inoculum consisted of 0.25 x 0.25 in. PDA squares, on which the organism had been grown for 4 days. These were placed into dehiscing fruits of Ne Plus Ultra on orchard trees. Sterile PDA squares were used in the controls. Each treatment was replicated 25 times.

Control treatments were negative, whereas 28% of the hulls inoculated with *Botrytis* were rotted; only 1 twig showed leaf blighting. The PDA possibly promoted colonization of the hull by *Botrytis*. Fruits inoculated with *Stemphylium* sp. and *Alternaria* sp. developed no detectable symptoms on fruit or leaves. In similar inoculations on Nonpareil trees, *Botrytis* sp. rotted 4 of 15 fruits, but produced no leaf blighting. *Rhizopus* sp., however, sporulated on 2 of the rotted fruit. Likewise, on 1 fruit which rotted after being inoculated with *Alternaria* and was followed by the blighting of the twig, *Rhizopus* sp. was found fruiting on the hull and was isolated from tissue of the attached fruit. Blighted twigs, with *Rhizopus* spp. fruiting on the hull of the attached fruit, occurred to some extent in the experimental orchard.

In a laboratory experiment, 5-week-old fruits of Peerless and Nonpareil almond on detached twigs were inoculated with a spore suspension of *R. stolonifer* and *M. fructicola*. Some fruits were inoculated with *R. stolonifer*, some with a combination of *R. stolonifer* and *M. fructicola*, and some were controls. The combination inoculation produced no more severe symptoms than *R. stolonifer* alone. This confirmed field data, indicating that *Rhizopus* spp. need not act as secondary invaders after infection by *M. fructicola* and *M. laxa*.

Distribution of the disease.—Almond hull rot occurs in all the major almond-growing counties of the Sacramento and San Joaquin valleys. A survey in the summer of 1957 revealed a high incidence in Sutter, Butte, and Stanislaus counties. The disease has not been reported from other almond-growing countries.

TABLE 1. Frequency of isolation and identity of fungi isolated from hulls, peduncles, and stems of almond

Fungi isolated	Hulls		Peduncle		Stem	
	NCO[a]	%F[b]	NCO	%F	NCO	%F
Botrytis spp.	20	13.5	3	4.8	0	0.0
Monilinia fructicola	29	19.5	7	11.3	0	0.0
Rhizopus spp.	63	42.5	19	30.6	11	11.9
Pullularia spp.	2	1.3	6	9.6	11	11.9
Alternaria spp.	24	16.2	4	6.4	3	3.2
Monilinia laxa	2	1.3	1	1.6	0	0.0
Aspergillus spp.	1	0.6	0	0.0	0	0.0
Penicillium spp.	1	0.6	0	0.0	0	0.0
Phomopsis spp.	2	1.3	0	0.0	0	0.0
Chaetomium spp.	7	4.7	0	0.0	0	0.0
Pleurage spp.	2	1.3	0	0.0	0	0.0
Nigrospora spp.	1	0.6	0	0.0	0	0.0
Cladosporium spp.	0	0.0	0	0.0	1	1.0
Fusarium spp.	3	2.0	0	0.0	0	0.0
Stemphylium spp.	9	6.0	0	0.0	0	0.0
Hormiscium spp.	1	0.6	1	1.6	2	2.1
Coryneum beijerinckii	1	0.6	0	0.0	0	0.0
Phoma spp.	1	0.6	0	0.0	0	0.0
Unidentified	3	2.0	0	0.0	0	0.0

[a] Number of cultures obtained.
[b] Percentage of frequency of isolation, based on 148 attempts from diseased hulls, 62 from peduncles, and 92 from twigs.

Fig. 5. The shoot situated above the rotted fruit represents current year's growth. The leaves on 1 side of the long axis of the shoot show unilateral necrotic lesions.

Host susceptibility.—Judged by observations over a 3-year period, all almond varieties are susceptible. Under natural conditions, however, both hull rot and twig blight are most severe on Nonpareil. Next in order of susceptibility are Jordanolo and Ne Plus Ultra. The hulls of Texas (Mission) and Ne Plus Ultra are usually rotted, with little blighting of the supporting twigs. Fruit of both varieties drops soon after rotting. Furthermore, the mesocarp of Texas fruit severs its connection with the peduncle at an early stage of dehiscence (3).

Time of infection.—The disease develops only after the hulls dehisce. Inoculation of the fruit apparently occurs when the suture of the mesocarp ruptures (Fig. 4), exposing the moist inner surface of the mesocarp to air-borne spores of the causal fungi.

Spores of *R. circinans* and *R. arrhizus* did not produce hull rot when placed on the surface of mature-sized but indehiscent Nonpareil fruit. Hull rot occurred, however, when the spores were placed in the open suture of dehiscing fruit.

Moisture content of almond fruits during dehiscence.—Under the environmental conditions prevailing in

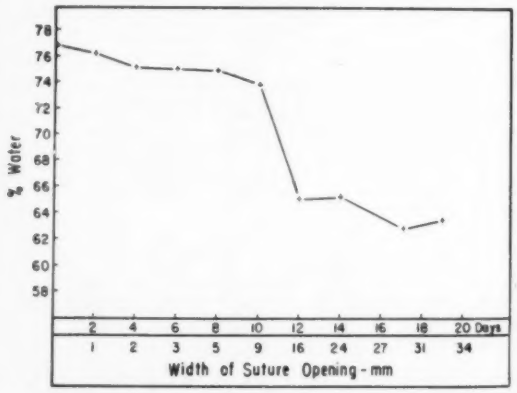

Fig. 6. Water content of almond hulls from the beginning to completion of dehiscence, expressed as percentage of dry weight.

the orchard during the summer of 1957, the moisture content of hulls remained between 74–77% (Fig. 6) from the beginning to completion of dehiscence. The inoculation tests, previously reported, showed that *Rhizopus* actively attacked and rotted almond hulls during this period.

DISCUSSION.—*Rhizopus* spp. have been considered weak parasites associated largely with the decay of fleshy or senescent plant tissue. This study shows them capable of producing, in shoots of woody plants, symptoms similar to those produced by the more aggressive parasites, *Monilinia fructicola* and *M. laxa*.

It is apparent from results of isolation and histological studies that *Rhizopus* does not induce the twig and leaf symptoms of the disease by direct invasion. Since extensive physical obstruction or disorganization of the xylem elements is not present, it can be concluded that leaves do not die because they are deprived of water. In fact, they do not develop the flaccid condition generally associated with an interruption of the water supply. Instead, an initial necrosis of 50% of the leaf blade often precedes death of the entire leaf. Consideration of these features and the local (unilateral) development of twig symptoms suggests that a toxin produced in the fruit mesocarp is responsible for twig and leaf symptoms. The search for the toxin and demonstration of the pathway through which it is translocated to the leaves is the subject of a later paper.

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EFFECT OF DUTCH ELM DISEASE ON SEEDLING ELMS

Toru Arisumi and Daniel J. Higgins

Respectively, Geneticist, Crops Research Division, ARS, U.S.D.A., Beltsville, Maryland; and Former Pathologist, Crops Research Division, ARS, U.S.D.A., Delaware, Ohio, now, Department of Biology, University of Dayton, Ohio.

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SUMMARY

Progenies of American, European, and Asiatic elm seedlings in their third growing season were artificially inoculated and evaluated for resistance to Dutch elm disease[*Ceratocystis ulmi* (Buis.) C. Moreau], according to the percentage of crown affected by disease. This statistic was directly estimated by the percentage of laterals, linear growth, and central leader affected by the disease; all were reliable estimates.

Statistical analyses of the results showed significant differences between progenies and between size groups within these progenies during the first growing season after inoculation. Although the larger seedlings developed more disease symptoms than the smaller ones during the summer, the latter showed greater injury during the winter. As a result, the seedlings compared for total disease injury 1 year after inoculation showed no significant difference due to size.

The growth of all inoculated seedlings, except the progeny of Netherlands 148 × *U. pumila*, was severely inhibited for 2 growing seasons. These were considered resistant to Dutch elm disease because of their low percentage of injury and their ability to maintain growth after inoculation.

INTRODUCTION.—The difficulty of inducing Dutch elm disease symptoms on seedling elms by artificial inoculation has been noted by several investigators (2, 3). Heybroek (3) mentioned juvenile resistance in 2- and 3-year-old seedlings. From his tests, Heybroek concluded that resistance is gradually diminished as seedlings mature and approach the flowering stage. He suggested that probably no further decrease occurs after the flowering stage.

False evaluation of resistance could result from conditions that prevent effective inoculation or suppress symptom expression. Banfield (1) found that the large spring vessels were more effective for spore distribution than vessels of the summer wood and that the extent of infection was correlated with vessel length at the point of inoculation.

Went (7) reported that trees inoculated soon after transplanting or grafting were not affected by Dutch elm disease. Heybroek (3) also observed that trees growing in certain areas in The Netherlands were less susceptible because of their slow growth.

Relatively few species of elm are resistant to Dutch elm disease. Extensive tests of American, European, and Asiatic elm species by Went (7) showed that

TABLE 1. The effect of Dutch elm disease on seedling progenies

Progeny	Caliper (in.)	Seedling inoculated (no.)	Seedling symptomless (no.)	Seedling affected (no.)	Crown affected in 1958		Crown killed by end of 1958-59 winter season (mean % central leader)
					Mean % laterals	Mean % linear growth	
Open pollinated Ky 40	¼	74	30	44	7.5	6.7	35.8
	½	120	22	98	17.1	16.8	40.9
	¾	33	1	32	34.0	45.5	49.3
Progeny avg					15.7	17.6	43.6
Amer. 1 × Ky 40	¼	450	105	345	12.1	10.1	47.9
	½	288	14	274	30.2	22.9	47.0
	¾	27	0	27	48.1	39.7	60.2
Progeny avg					19.9	15.0	47.9
Ky 40 Selfed	¼	6	0	6	22.8	14.1	24.6
	½	23	0	23	48.6	28.1	46.2
	¾	3	0	3	71.1	42.0	58.7
Progeny avg					45.6	26.4	46.4
Fulva 1 × Pumila	¼	13	4	9	6.0	3.1	71.0
	½	20	4	16	7.2	4.9	54.9
	¾	7	1	6	19.4	14.2	57.0
Progeny avg					8.5	5.5	57.2
Fulva 2 × Pumila	¼	14	7	7	2.6	2.4	45.0
	½	40	6	34	8.0	5.2	41.1
	¾	55	1	54	15.9	12.6	33.3
Progeny avg					11.3	9.8	38.6
Amer. 1 Selfed	¼	3	0	3	17.6	5.9	35.9
	½	6	1	5	27.2	12.9	58.8
	¾	0					
Progeny avg					23.8	10.4	53.6
Neth. 148 × Pumila	¼	2	1	1	2.5	1.5	0[a]
	½	7	5	2	1.2	0.7	0
	¾	0					
Progeny avg					1.4	0.2	0[a]
Amer. 1 × Moline	¼	5	2	3	8.4	8.7	39.5
	½	5	0	5	27.2	23.7	53.3
	¾	0					
Progeny avg					16.8	16.0	49.2
Fulva 2 Selfed	¼	4	4	0	0	0	50.1
	½	12	7	5	1.3	0.7	36.0
	¾	3	1	2	7.5	2.0	24.2
Progeny avg					1.3	0.5	36.0
Moline × Ky 7	¼	4	0	4	18.1	9.5	70.8
	½	8	0	8	28.4	22.1	59.5
	¾	0					
Progeny avg					24.7	17.4	60.8
Moline × Ky elm	¼	2	0	2	36.0	17.6	36.99
	½	7	0	7	25.7	14.9	42.6
	¾	0					
Progeny avg					27.9	15.4	40.0
Significant Mean Differences at 5% level							
Between progenies					2.8	2.5	3.3
Between size groups within progenies					24.8	21.3	NS

[a]According to height measurements the seedlings in this progeny showed 8.6% net gain in top growth.

certain Asiatic elms, such as *Ulmus pumila* L., *U. pumila* var. *pinnato-ramosa* (Dieck) Henry, and *U. parvifolia* Jacq., were highly resistant. *U. americana* L. and most European species were very susceptible. Walter (6) also found that *U. americana* was more susceptible than the European elms to Dutch elm disease. According to Went (7), the use of resistant trees for breeding with susceptible trees showing desirable growth form has raised the level of resistance in the hybrids.

In preliminary tests with 1-year-old seedling elms artificially infected with Dutch elm disease, the array of seedlings, classified according to the estimated percentage of crown affected by disease, could be analyzed by standard statistical procedure. The seedlings also showed quantitative differences, indicating a probable polygenic inheritance for resistance to Dutch elm disease. From these results, it seemed feasible to study quantitative methods of evaluating disease resistance on young, field-grown elms.

MATERIALS AND METHODS.—Elm seedlings in their third growing season were used for these tests. They were obtained from breeding experiments conducted by the senior author in winter and early spring of 1956. The parental trees are identified as follows: Ky 40, Ky 7, and Ky 9 are selections of *U. americana* L. from central Kentucky; Amer. 1 and Moline are selections of *U. americana* L.; Fulva 1 and Fulva 2 are

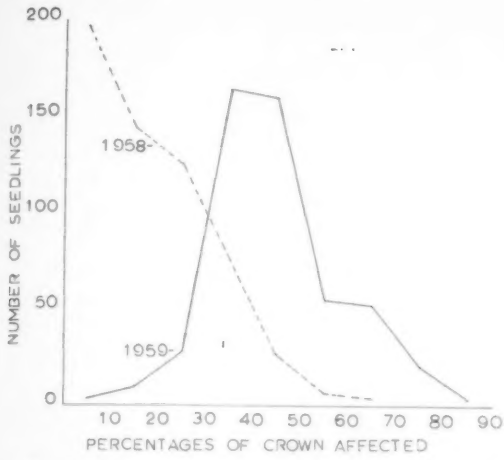

Fig. 1. Frequency distribution of 0.25-in. diameter seedlings according to the percentages of crown affected before winter (1958) and after winter (1959).

selections of *U. fulva* Michx; Pumila is a selection of *U. pumila* L.; and Netherlands 148 (*U. hollandica* Mill. Var. *vegeta* (Loud.) Rehd. × *U. carpinifolia* Gleditsch) is a Dutch elm disease-resistant selection from The Netherlands. All parental trees, except those from central Kentucky, were obtained locally from the shade tree nursery of the U.S. Department of Agriculture or from the vicinity of Columbus, Ohio.

The seedlings were inoculated with a spore suspension of *Ceratocystis ulmi* (Buis.) C. Moreau, on May 26, 1958, when the spring vessels were open. The inoculation date was chosen by periodically following the development of spring vessels using Banfield's (1) dye-injection method. Each seedling was inoculated by direct introduction of 0.5–1.0 ml spore suspension into the sap stream through a knife wound made at the base of the stem. The spore suspension was prepared by shake-incubating the fungus in potato-dextrose broth. Spore count at the time of inoculation was 10^7/ml.

Two weeks after inoculation, when disease symptoms were apparent in the test rows, the symptomless individuals were labeled. They were examined periodically during the growing season. Some developed symptoms later and were included in the symptomatic group. All seedlings were measured for height, stem caliper, and extent of crown injury after symptoms had attained their maximum.

Comparisons were based on the percentage of crown affected by foliar wilt or die-back. This statistic was estimated for each seedling by 3 methods during the course of the experiment. In the summer of 1958, it was estimated by the percentage of laterals and the percentage of their linear growth affected by disease. The percentage of laterals affected was computed from the total number and the number diseased. The percentage of linear growth affected was computed from the lengths of the diseased portions in relation to the total lengths of these laterals. For these 2 methods, only laterals longer than 6 in. were used. In 1959, the percentage of central leader die-back (4, 6) was used as an estimate of the percentage of the crown killed by disease.

Each progeny was divided into subgroups classified according to stem caliper. The data were pooled and analyzed for statistical significance by analysis of variance after transformation to angles. Where applicable, the significant mean difference at the 5% level was computed according to Snedecor's (5) modification of Tukey's method.

Results.—Disease symptoms were apparent 1–2 weeks after inoculation. At first, the tips of the twigs began to droop and wilt, followed by further wilting and die-back of successively older portions of the twigs. The symptoms became progressively more pronounced as discoloration and shriveling commenced on the affected sections of the crown. Although intensification of symptoms continued until early August, the extent of crown affected by disease became maximum by the end of June.

Reaction to the disease ranged from symptomless plants (column 4, Table 1) to those having over 90% of the crown affected. None of the seedlings, however, was killed during the first growing season. In most groups, the mean percentage of crown affected was higher when calculated by the percentage of laterals showing symptoms than when calculated by percentage linear measure. This difference was due to the preponderance of seedlings showing large numbers of slightly affected laterals and is reflected in the difference between comparable percentages in columns 6 and 7, Table 1. Significant mean differences for each method are listed at the end of their respective columns.

All seedlings except Netherlands 148 × *U. pumila* showed no linear growth after inoculation. Control groups showed height gains of 1.0–1.5 ft. Development of fall leaf colors indicated that the control and inoculated seedlings became dormant at about the same time. Both were completely defoliated by the end of October.

By the early spring of 1959, most of the inoculated seedlings had incurred extensive die-back during the winter. This die-back extended into the older wood of primary laterals and central leaders apparently unaffected during the summer. Winter injury was not observed among the controls. The mean cumulative percentages of crown killed by early summer of 1959 are shown in the last column of Table 1. Only 20 of 216 inoculated but symptomless seedlings survived without winter injury. The remainder of the symptomless seedlings showed average crown injury only slightly less than the average of their respective groups. Of 1,025 diseased seedlings, only 8 were killed during the winter.

Fig. 1, 2, and 3 show the comparative distributions of seedlings of different calipers according to percentage of crown affected in 1958–59. The percentage

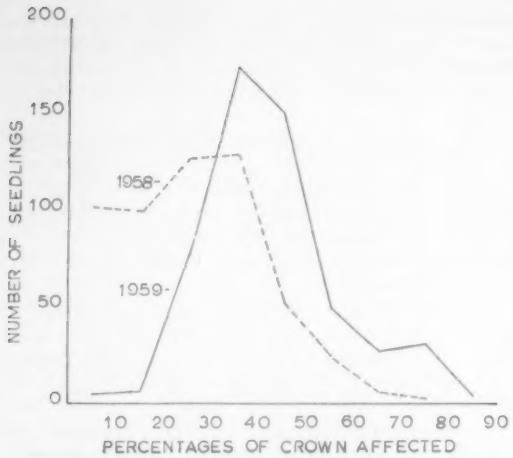

Fig. 2. Frequency distribution of 0.5-in. diameter seedlings according to the percentages of crown affected before winter (1958) and after winter (1959).

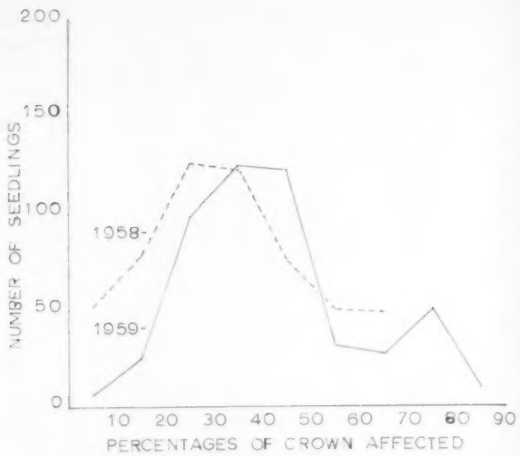

Fig. 3. Frequency distribution of 0.75-in. diameter seedlings according to the percentages of crown affected before winter (1958) and after winter (1959).

obtained by linear measure was used for plotting the 1958 distributions shown in the figures. For the 0.75-in. caliper group (Fig. 3), the actual frequencies were proportionately increased to that of a theoretical population of 500 seedlings for comparison with the 0.25- and 0.5-in. caliper groups (Fig. 1, 2), on equal scale. The frequency polygons were plotted along the midpoints of classes with a 10% class range. Comparisons of Fig. 1, 2, and 3 show that, after inoculation, the smaller seedlings were more severely damaged by winter than the larger seedlings.

No recurrence of the disease was observed during the growing season of 1959. Seedling growth was inhibited with little or no recovery of top growth lost during the preceding year. Part of this slow growth could have been due to drought in the early summer as the growth of controls was somewhat restricted. Height gains of 0.5–1.0 ft were recorded for the controls in 1959.

DISCUSSION.—Reliable estimates of the percentage of crown affected by disease were obtained by the 3 methods of measurement used in this study. Estimating by measuring the central leader is most practical for testing large numbers of seedlings. This method, however, is limited to populations showing extensive dieback or to seedlings more than 0.75-in. in caliper. For close comparisons among seedling groups, linear measure of laterals is best. Estimating the percentage of laterals affected is less accurate, but not as time consuming, as making linear measurements. The variances of these 3 methods were small and did not differ significantly.

The results showed that seedlings in the 0.75-in. group were more susceptible to the disease than the smaller seedlings during the first growing season after inoculation. For this reason, tests of extremely young seedlings may result in false evaluation of resistance. The higher degree of resistance shown by smaller seedlings during the summer might have been due largely to lower transpirational losses. Besides having sparse top growth, the smaller seedlings had the added advantage of being partially shaded by the larger ones in the test row.

The growth-inhibiting effect of Dutch elm disease should have an important bearing on continued testing of elm selections. If stunting persisted for extended periods, repeated inoculation over successive years might not produce disease symptoms.

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ROLE OF FUMARIC ACID IN THE HULL ROT DISEASE OF ALMOND

C. J. Mirocha, J. E. DeVay, and E. E. Wilson

Department of Plant Pathology, University of California, Davis.

Respectively, Plant Pathologist, Union Carbide Research Station, Clayton, North Carolina; and Associate Professor and Professor, Department of Plant Pathology, University of California.

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SUMMARY

Rotting of the hulls (mesocarp) of almond fruit by *Rhizopus stolonifer*, *R. circinans*, and *R. arrhizus* is often accompanied by unilateral necrosis of the leaves on the same side of the twig as the infected fruit. Since mycelium of the fungus does not extend into the twig, a toxin, originating in the rotted fruit, was identified as the agent responsible for twig and leaf symptoms.

A natural pathway through which a toxin might move from the fruit to the leaves was demonstrated with radioactive phosphorus. This material moved readily out of the hulls of dehiscent, infected, and uninfected fruit; and accumulated in the leaves in a manner similar to the distribution of leaf symptoms.

C^{14}-labeled mycelium of *Rhizopus*, inoculated into almond fruits on orchard trees, produced symptoms in twigs and leaves identical to those produced by natural infection. Moreover, radioautographs showed a unilateral distribution of C^{14} in stems and leaves corresponding to the unilateral distribution of symptoms. Controls with killed mycelium were negative. When certain metabolic products of *Rhizopus* were injected into fruits on orchard trees, tests demonstrated that fumaric acid, in concentrations usually present in diseased fruits, would cause the disease symptoms in leaves and twigs. Additional tests with C^{14}-labeled fumaric acid showed that, before leaf and twig symptoms were apparent, fumaric acid was translocated to the shoot and accumulated in the leaves. During symptom development, however, radioactive fumaric acid rapidly disappeared and the radioactive carbon was present in malic, citric, and tartaric acids. Moreover, fumaric acid was not detected in healthy leaves, whereas malic and citric were present in relatively large amounts. In contrast, 0.2–2.3 mg fumaric acid/g dry wt occurred in diseased hulls; but only trace amounts occurred in healthy hulls. It was concluded that fumaric acid produced in rotted hulls by *Rhizopus* is translocated to twigs and leaves, where it or some of its metabolic derivatives are highly toxic.

INTRODUCTION.—Hull rot is a disease of almond (13, 14) that can be caused independently by *Rhizopus* spp., *Monilinia fructicola* (Wint.) Honey, and *M. laxa* (Ader. & Ruhl.) Honey. The disease is characterized by colonization of the fungus on the interior of the fruit hull and subsequent blighting of the adjoining twig and its leaves. The mesocarp (hull) of the fruit is rotted and the vascular tissue of the adjoining twig develops a brown to black discoloration on the same side of the stem as the diseased fruit. Leaves on the same side of the twig as the rotted fruit often develop unilateral necrotic areas, then curl and become brittle. Infection takes place in midsummer at dehiscence of the fruit mesocarp.

Frequent isolations and examinations of tissue sections showed that the fungi do not invade the adjoining twig. Evidently, twig and leaf symptoms are induced by means other than direct invasion by these fungi. This suggested that a toxin is produced in the rotted hulls and transported to the twigs and leaves.

The present study was concerned with the identification of the toxin, proof of its ability to produce the leaf and twig symptoms, and the pathway through which it reaches the leaves.

MATERIALS AND METHODS.—Radioactive phosphorus was used to study the translocation pathway from dehiscent and indehiscent fruit to twigs and leaves. Approximately 2 μc of $H_3P^{32}O_4$ in 10μl of water were applied to fruits of the varieties Ne Plus Ultra, Nonpareil, and Peerless on orchard trees. The isotope was placed via a micropipette into a well (0.125 in. deep x 0.125 in. diameter) cut into the side of the hull midway between its apex and base (Fig. 2-A). The twigs were harvested after 4, 27, and 52 hours. The shoots minus the fruit then were wrapped in Saran plastic and autoradiographed in a press held at −10°C. Radioactivity in counts/minute (CPM) was determined by scanning the leaves with a Tracerlab TGC-2 thin-end window Geiger-Mueller tube attached to a Tracerlab Autoscaler.

All extractions of plant materials were made with water or 70% ethanol. The material was air-dried and then homogenized with the extracting solution for 5 minutes in a Waring Blendor. Twenty ml of extractant were used/g dry wt of tissue. The tissue was extracted 3 times. In each successive extraction, the amount of extracting solution was reduced 50%. Between transfers of the tissue, the glassware was rinsed 4 times. The solutions were combined and concentrated at less than 35°C to 5–25 ml in a glass evaporator (17). The concentrated extract was then passed through an Amberlite IRA-400 resin column, as described by Bryant and Overell (2, 3). Compounds adsorbed on the resin were eluted with 300–400 ml of 1*N* $(NH_4)_2$-

CO_3. After elimination of the NH_4^+ and $CO_3^=$ by heating on a steam bath, the extract was analyzed by both paper and gas chromatography (12). The latter method, however, did not require desalting with an ion-exchange resin.

The paper chromatographic method used was essentially that described by Bryant and Overell (2) and Lugg and Overell (10). Where paper chromatographic identification was necessary, 2 solvent systems were used: 75:17:60 v/v butanol, formic acid, and water; and 75:75:36 v/v reagent grade mesityl oxide, water, and 90% formic acid. Known organic acids, used in identifying unknowns, were purchased from the California Corporation for Biochemical Research.

Almond fruit on orchard trees was treated with metabolites of *Rhizopus* in the following manner: the fruit chosen was usually adjacent to a current year's shoot, so unilateral symptom development could be followed easily. With a 0.25-in. cork borer, a well was made in the mesocarp, extending up to 0.75 of the way through. A glass tube of 0.187 in. diameter, with a capacity of about 3.5 ml, was inserted a short distance into the well and sealed to the fruit with paraffin (Fig. 5). Three ml of the test metabolite then were pipetted into the tube and taken up by the fruit. Distilled water then was added to the tube.

In experiments where C^{14}-2,3-fumaric acid was used, 25 μl of water with 2.5 μc of the acid were introduced into the tube after 3 ml of the unlabeled fumaric acid (0.025-0.05*M*) had been placed in it. The control fruits, on another shoot of the same branch, were treated with water at the same pH as the test solution.

Rhizopus mycelium, used in this experiment as inoculum, was labeled by allowing the organism to grow on labeled C^{14}-glucose, C^{14}-fructose, and a mixture of other C^{14}-labeled substances in extracts of *Canna* leaves.

The radioactive compounds were synthesized by a modified method of Putnam and Hassid (15, 16). Excised leaves of *Canna indica* L. were placed in a 15.5-liter glass cylinder containing 7.8 mc of $BaC^{14}O_3$ (specific activity, 0.061 mc/mg). $C^{14}O_2$ was liberated by addition of 3 ml of 0.1*N* HCl solution, which gave a concentration of about 0.1% CO_2 in the atmosphere of the sealed chamber. The leaves were continuously illuminated by 2 incandescent 100 w bulbs in the greenhouse; light intensity varied between 400 ft-c at night and 960 ft-c during the day. The temperature inside the chamber fluctuated between 24° and 30°C for the duration of the CO_2 fixation period (27.5 hours). The leaves then were extracted with 80% ethanol using 10 ml/g fresh weight of tissue. After the excess pigments were removed with diethyl ether, the alcohol extract was concentrated in a glass evaporator (17) and made to a volume of 20 ml. Five mg of invertase (Nutritional Biochemicals Corporation) were added to the concentrated extract and incubated for 30 minutes to hydrolyze sucrose, since sucrose is not metabolized by *Rhizopus* spp. Ten ml of this radio-

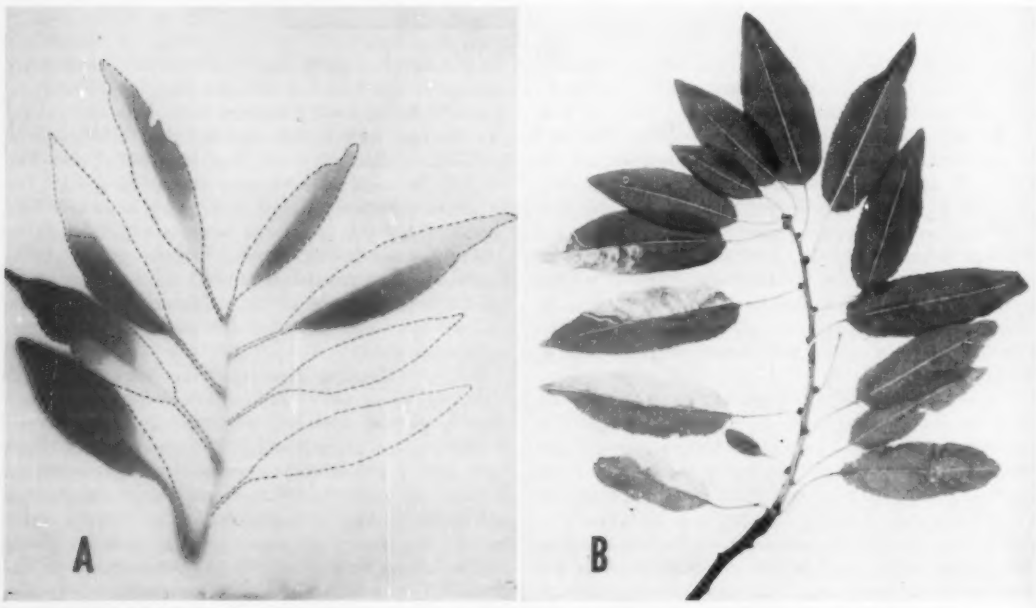

Fig. 1. A) Distribution of radioactive phosphorus in the leaves of almond after inoculation with P^{32} into fruit on the adjoining shoot. **B)** Symptoms due to natural infection of the fruit. Both shoots are of current-year growth and the P^{32}-inoculated and *Rhizopus*-rotted fruits were located on the same side of the stem as the accumulation of radioactivity and disease symptoms occurred.

TABLE 1. Translocation of P^{32} out of healthy almond fruits of Nonpareil and Peerless at various stages of dehiscence

Fruit no.	Leaf distance[a]	Suture opening (mm)	Translocation
1[c,d]	At peduncle	45	+
	Side shoot, 2 in. below		+
	3 in. below		+
	4 in. below		+
	5 in. below		−
1P[b]	3 in. above	10	+
	1 in. above		+
	1.5 in. below		+
2[b]	0.5 in. above	10	+
4[b]	At peduncle	8	+
	3.5 in. above		+
5[c]	At peduncle	5	+
	1 in. below		+
	2.5 in. below		+
10[c]	At peduncle	8	+
	2.5 in. below		+

[a] Distance of autoradiographed leaves from the peduncle of the inoculated fruit.
[b] Inoculated fruits intercalary on the stem.
[c] Inoculated fruits terminal on the stem.
[d] Mesocarp of fruit attached to peduncle by only 1 side.

active extract then were added to 15 ml of the following nutrient solution:

$MgSO_4 \cdot 7H_2O$	3.0 g	H_3BO_3	0.06 mg
NH_4Cl	3.0 g	$CuCl_2 \cdot 2H_2O$	0.05 mg
KH_2PO_4	3.0 g	$MnCl_2 \cdot 4H_2O$	0.05 mg
$FeCl_3 \cdot 6H_2O$	0.24 mg	$NaMoO_4 \cdot 2H_2O$	0.03 mg
$ZnCl_2$	0.15 mg	D-glucose	10.0 g

Distilled water to make 1 liter

The medium was placed in a 125-ml Erlenmeyer flask, autoclaved, and seeded with 2 ml of a spore suspension of *Rhizopus circinans* v. Tiegh. The culture was sealed with polyethylene and incubated on a reciprocating shaker for 72 hours at 23–27°C. A highly radioactive mycelial preparation resulted.

Uniform inoculum for field inoculations was obtained by pouring the culture on filter paper (previously marked into 0.25-in. squares) in a Büchner funnel and distributing the mycelium evenly on the surface of the paper. Mycelium from each square then was peeled off and used as inoculum. The control inoculum was killed by steaming for 5 minutes.

Fruit on orchard trees was inoculated by placing the mycelium in a hole cut in the mesocarp with a cork borer, and replacing the plug of tissue.

Relative amounts of radioactivity in the leaves were indicated by autoradiographs and scanning the leaves with a Tracerlab TGC-2 thin-end-window Geiger-Mueller tube attached to a Tracerlab Autoscaler. Radioactivity in C^{14}-labeled organic acids was determined by eluting the acids off paper chromatograms with 70% ethanol or water into scintillation counting vials and then evaporating the solution to dryness. Then, 0.1 ml of $10^{-2}M$ phemerol chloride (Hyamine 1662) in toluene was added to increase the solubility of the acids in the scintillation solvent [10 ml of 0.6% 2,5 diphenyloxazole (PPO) and 0.05% 1-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in toluene]. Counts were made in a Tri-Carb Liquid Scintillation Spectrometer.

Autoradiographs of plant materials were made by placing fresh tissues in an envelope of Saran wrap (Dow Chemical Co.) backed with cardboard. A piece of Kodak No-screen Medical X-ray film was placed between the cardboard and the envelope and exposed in a Kodak film holder at −14°C.

EXPERIMENTAL RESULTS.—*Origin of leaf and twig symptoms.*—Search for a toxin was preceded by determination of whether a pathway for translocation of a toxin from the rotting fruits to the leaves was present during the time of dehiscence. A pathway was demonstrated by placing radioactive phosphorus in the fruit and detecting its movement into the adjoining twig and leaves. Radioactive phosphorus was chosen as the tracer because of its capacity of being translocated in either phloem or xylem (1, 4) and predominantly as phosphate ion (19, 20).

Comparison of the distribution of P^{32} in almond leaf-shoot with distribution of disease symptoms.—On August 20, 1958, 2 μc of $H_3P^{32}O_4$ in 10 μl of solution were applied to Ne Plus Ultra fruit in an orchard. The mature fruits chosen were those at the base of a current year's shoot; their sutures had opened about 4 mm. The twigs were harvested after 27 and 52 hours and autoradiographed for 53 hours.

Radioactive phosphorus was readily translocated from fruit into the adjoining shoot. Its distribution between leaves was unilateral, *i.e.*, it tended to accumulate more in leaves on the same side of the stem as the fruit, and in 1 side of the leaf blade (Fig. 1-A).

In some cases, the pattern of distribution resembled that in Fig. 2. While radioactivity was greatest in leaves on the peduncular side of the stem, some activity occurred in leaves on the opposite side. More important, however, was the unilateral accumulation of P^{32} in the leaf blade, closely resembling the unilateral necrotic areas produced by the disease on current year's shoots and leafy spurs (Fig. 1-B).

Translocation of P^{32} out of mature almond fruits at different stages of dehiscence.—On August 18, 1958, Nonpareil and Peerless fruits on 6-year-old trees were treated with radioactive phosphorus as already described. About 3–4 μc of $H_3P^{32}O_4$ were applied to each fruit. Healthy fruits in various stages of dehiscence and dehiscing fruit infected with *Rhizopus* were chosen to test translocation of P^{32} from fruit to

TABLE 2. Translocation of P^{32} out of *Rhizopus*-infected dehiscing fruit

Fruit no.	Distance of leaf from fruit	Translocation
3	1.5 in. above	+
	4.0 in. above	+
5A	4.0 in. above	+
	7.0 in. above	+
8	At peduncle	+
	1.0 in. above	+
	12.0 in. above	+

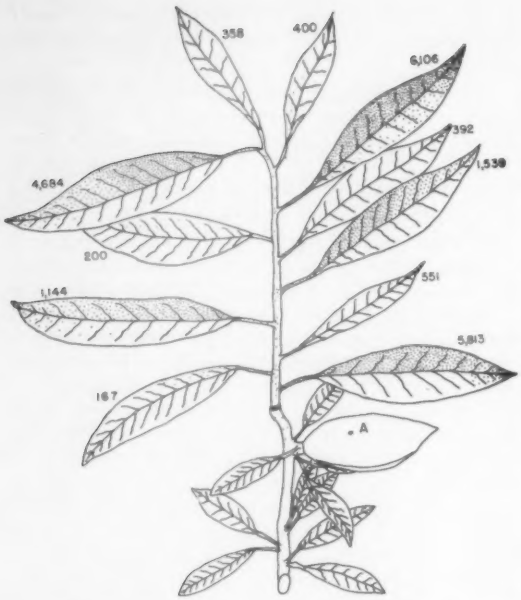

Fig. 2. Discrete unilateral pattern of P^{32} distribution and accumulation in the leaf lamina when applied to the Nonpareil fruit at point A. The density of the stippled areas represents the degree of radioactivity as determined by an autoradiograph. Numerical values are in counts/min.

leaves at the time of disease development. In addition, fruits borne both terminally and intercalary on the stem were inoculated to ascertain if fruit position affected translocation. Shoots were harvested 25 hours after treatment and leaves were autoradiographed for 19 days. The developed film showed that translocation of P^{32} had occurred from all of these fruits (Table 1, 2).

The method of dehiscence of Nonpareil fruits and of translocation of P^{32} are of particular interest. Initially, the suture of the mesocarp opens and the vascular connection between the hull and the peduncle remains intact on both sides. At full dehiscence, the vascular connection often severs on 1 side but remains intact on the other (Fig. 3). Radioactive phosphorus applied to the side of the mesocarp with the intact vascular connection moved readily into the stem, whereas translocation from the opposite side was negligible.

In these and other experiments, P^{32} consistently accumulated selectively in leaves situated on the same side of the stem as the attachment of the peduncle of the inoculated fruit. In some cases, where all 5–6 leaves of a single cluster were autoradiographed, a large amount of radioactivity was found in some, and little or none in others. This suggests that the vascular connection from the inoculated fruit was restricted to the radioactive leaves.

P^{32} was translocated both upward and downward in the stem from the point of attachment of the inoculated fruit. More often, however, it was translocated much farther upward than downward.

Toxic metabolites in culture filtrates.—A water extract of healthy almond hulls (var. Ne Plus Ultra), previously stored in a freezer, was sterilized by filtration with an HA sterilizing Millipore filter. Seventy-five-ml portions were placed in 250-ml Erlenmeyer flasks and inoculated with a spore suspension of *Rhizopus* isolate No. 202. The stationary cultures were incubated for 13 days at 23–27°C. The culture medium then was resterilized by filtration and divided into 3 lots. Lot 1 was mixed "batchwise" with an Amberlite resin [IR-4B (OH) anion exchanger]; lot 2 was mixed with Amberlite resin [IRC-50, cation exchanger]; and lot 3 was not treated. One g of the resin was used to every 10 ml of the culture medium. After treatment, the medium was clarified and resterilized by filtration. Three lots of an uninoculated portion of medium that served as controls were treated as above. An additional control using sterile distilled water also was used.

Three-ml amounts of each test solution obtained above were placed in sterile glass vials within sterile 125-ml Erlenmeyer flasks capped with Whatman No. 1 filter paper and polyethylene. The basal 3 in. of young Nonpareil almond shoots (8–10 in. long) were surface-sterilized with 1:1,000 $HgCl_2$ solution for 3 minutes and rinsed 3 times with sterile distilled water. The shoots then were inserted into the vials containing the test solutions and incubated under diffuse laboratory light at 23–27°C. Treatments were replicated 6 times.

Within 16 hours, the leaves of 8 shoots of lot 3 (untreated) showed a cupping, twisting, and general brittleness; the midveins and lateral veins and some of the petioles turned dark brown; and the vascular tissue of the twig became necrotic. The shoots of lot 1 (treated with anion exchange resin) showed no symptoms, *i.e.*, they did not differ from the water controls. Shoots of lot 2 (treated with cation exchange resin) showed the symptoms described for shoots of lot 3 (Fig. 4). None of the above symptoms was evident in any of the control shoots until 40 hours later. Then,

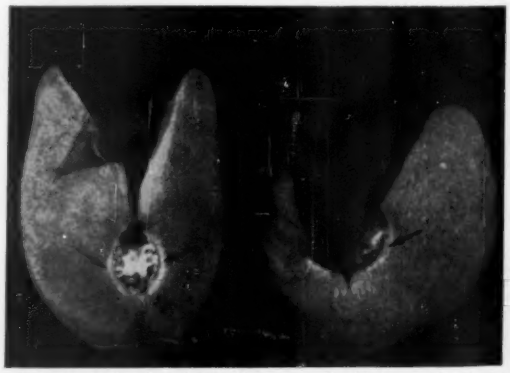

Fig. 3. Dehiscing hulls of Nonpareil. Hull at left shows intact vascular connection as it attaches to peduncle. At right, only 1 side remains intact. P^{32} readily moves out of the fruit when inoculated into that side of the mesocarp which has an intact vascular connection.

Fig. 4. Results 24 hours after shoots of almond had been inserted into vials containing sterile culture filtrate of the almond hull medium from a 13-day-old culture of *Rhizopus circinans*, isolate No. 202. Left to right, untreated, medium treated with cation exchange resin, and medium treated with an anion exchange resin. Shoots developed symptoms only where the anion fraction was present.

2 of the shoots inserted into the control medium treated with the cation exchange resin showed a slight curling of lower leaves and mild veinal necrosis.

The toxin in the inoculated medium was probably anionic because no symptoms developed in shoots treated with the extract adsorbed with the anion exchange resin, whereas shoots treated with extract adsorbed by the cation exchange resin showed symptoms. The symptoms resembled typical leaf and shoot symptoms found in the orchard on diseased shoots bearing fruit infected by *Rhizopus*.

Paper chromatographic analyses of the test solutions revealed that fumaric acid was present in large amounts, and succinic acid in trace quantities, in lots 2 and 3. Neither of these acids was detected in the controls nor in lot 1. Citric and malic acids appeared in equal amounts in both experimental and control media. Thus, fumaric acid was present in appreciable amounts in the inoculated medium and not in the uninoculated control.

Since the toxic principle was anionic, certain metabolites identified in this fraction were tested for toxicity.

Toxicity of Fumaric Acid to Almond Shoots.—*Laboratory tests.*—Tests were made of the toxicity to almond shoots of 0.05 *M* solutions of potassium fumarate (pH 4.1) and sodium succinate (pH 7.5). So far as possible, manipulations were carried out under aseptic conditions. Current-year shoots (8–10 in. long) of Nonpareil almond seedlings were inserted into Erlenmeyer flasks as previously described, and treatments were replicated twice.

Within 21 hours, leaves on shoots treated with potassium fumarate showed a marked curling and twisting and a dark-brown discoloration of their mid- and lateral veins. Necrosis also was visible in some leaf petioles. The plants treated with sodium succinate showed no symptoms and did not differ from controls in water. The symptoms caused by potassium fumarate resembled those of naturally blighted shoots and of shoots treated with culture filtrates of *Rhizopus* grown in the almond-hull medium.

In a similar experiment, shoots of Nonpareil, Peerless, Ne Plus Ultra, Jordanolo, Drake, and Texas almonds collected in the orchard were tested for their reaction to 0.05 *M* fumaric acid (pH 2.3) and 0.05 *M* potassium succinate (pH 7.4). The controls consisted of water adjusted with KOH or HCl to pH 2.3 and pH 7.4. Treatments were replicated 3 times.

The fumaric-acid-treated plants showed symptoms within 20 hours and within 48 hours showed a pronounced vascular necrosis of the stem and dark discoloration of the leaf veins. A gum-like exudate was present on the stems. The plants treated with potassium succinate showed no symptoms. Shoots in water at pH 2.3 were healthy, except for necrosis in the basal 1 in. of the stem. The stem necrosis, however, found in plants treated with fumaric acid extended the entire length of the shoot. Leaves of the shoots in water at pH 7.4 were healthy, and there was no stem necrosis.

No varietal differences were found in the symptom severity induced by fumaric acid in the laboratory; the varieties Texas and Ne Plus Ultra, however, required 24 hours longer to develop symptoms as severe as those in other varieties.

Further laboratory experiments, with both current and second-year shoots of Nonpareil, revealed that 3 ml of fumaric acid in concentrations as low as 0.0125 *M* caused necrosis and water soaking of vascular tissue extending the entire length of the stem.

Toxicity tests in the orchard.—Adequate demonstration of the role of fumaric acid in disease development required production of the disease symptoms with purified toxin concentrations similar to those in affected tissues during natural disease development. Moreover, it was necessary to show that the toxin was effective at the stage of maturity that almond fruit normally reaches when the disease occurs, *i.e.*, at dehiscence of fruits. Since the hull was suspected as the site of fungal toxin production, it was necessary to show translocation of toxin from fruit to the adjoining twig and leaves.

For the most part, fruits chosen for treatment were borne on leafy shoots, thereby allowing observation of unilateral symptom development. The fruits were treated either at initial dehiscence or when the suture opening was about 2–3 mm wide. Both the salts and free acids of fumaric and succinic acids (0.05 *M*) were tested. The test solutions were pipetted into glass tubes inserted in the fruit mesocarp. From 15 to 35 mg of each substance were applied to the fruit; however, a small fraction of the materials in the surface film crystallized on the inner walls of the glass tubes after the fruit took up the solutions. As before, the control was water adjusted to the pH of the respective test solutions (pH 2.2, 4.2). Eight separate experiments were performed.

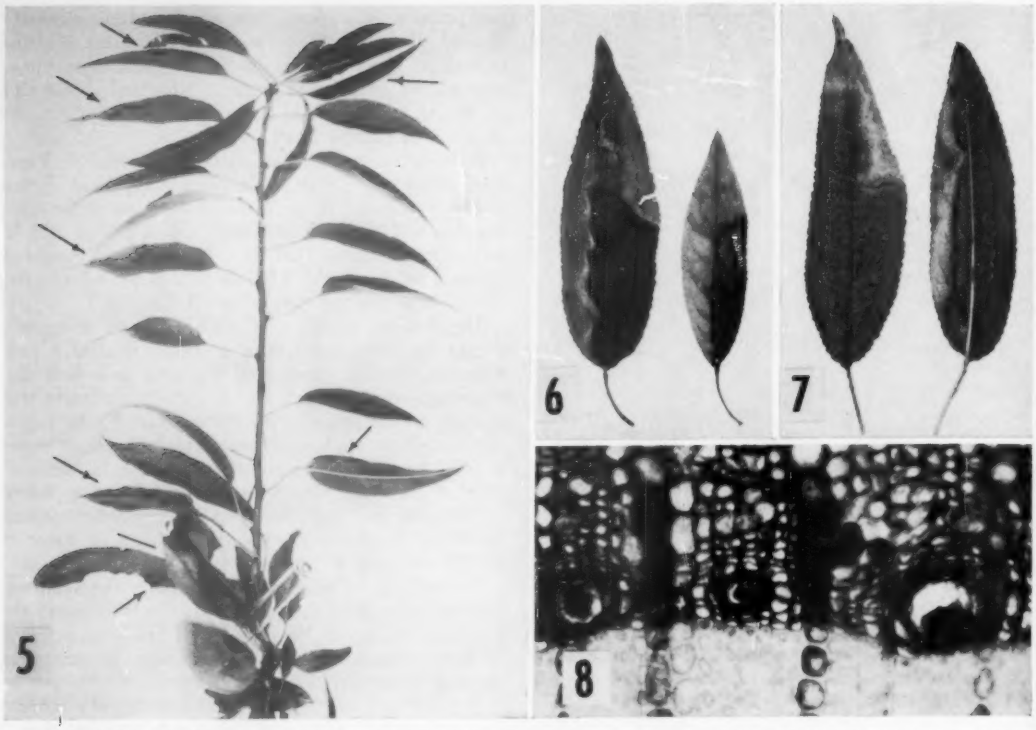

Fig. 5–8. 5) Unilateral necrotic leaf symptoms in the current-year shoot of almond (Texas) produced by inoculating the fruit with 3 ml of 0.05 *M* fumaric acid. Note that symptoms develop almost exclusively in leaves on the same side of the twig as the inoculated fruit. **6)** Necrosis in leaves due to natural infection of adjoining fruit and **7)** due to inoculation of fruits with fumaric acid. **8)** Transverse section of almond stem showing gum pockets in the young vascular tissue resulting from inoculation of the nearest fruit with fumaric acid.

Fumaric acid toxicity, evidenced by symptoms in shoots and leaves, was present in all 8 experiments. The symptoms, resembling those in natural infection, were evident 1–9 days after application of fumaric acid or its salt. Some leaves showed a light to dark-brown discoloration of the veins and petiole; marginal cupping, twisting, and brittleness also occurred. Many leaves developed unilateral necrosis typical of that found in leaves of current-year shoots having fruits rotted by *Rhizopus* (Fig. 6). A point of special significance was the development of symptoms in leaves with the same vascular connection as the treated fruit, *i.e.*, on the side of the stem of the fruit attachment (Fig. 5). Moreover, unilateral vascular discoloration developed on the same side of the stem as the inoculated fruit. Vascular bundles of the treated half of the mesocarp at their point of attachment to the peduncle showed a marked necrosis. Thus, the symptoms and their pattern of distribution duplicated that induced by natural infection with *Rhizopus*.

Histologic sections of the stems revealed in the young xylem tissue adjacent to the cambium, gum pockets, or ducts (Fig. 8) resembling those caused by natural infection. Like the leaf symptoms, the gum pockets were limited to the side of the stem to which the treated fruit was attached.

Succinic acid and its salt as well as the water controls (pH 2.2, 4.2) produced no apparent symptoms under these conditions.

PRODUCTION OF FUMARIC ACID IN THE DISEASE SYMPTOMS WITH RHIZOPUS MYCELIUM LABELED WITH C^{14}.—In 1911, F. Ehrlich (6) reported that *Rhizopus stolonifer* produced large amounts of fumaric acid. Since then, species of *Mucor*, *Cunninghamella*, and *Circinella* have been found to produce this acid in substantial amounts (7). Strains of *R. stolonifer* produce industrially important quantities of fumaric acid on certain media (9, 21). Production of significant amounts of fumaric acid by *Rhizopus* spp., grown on a medium containing a water extract of almond hulls as the sole carbon source, has been reported earlier (12). Thus, experiments were made to determine if the toxin (fumaric acid) is of fungal origin and if it is translocated from the fruit to the site of toxic action in the shoot and leaves.

R. circinans was grown in a nutrient solution containing various organic substances including glucose and fructose that were randomly labeled with C^{14}.

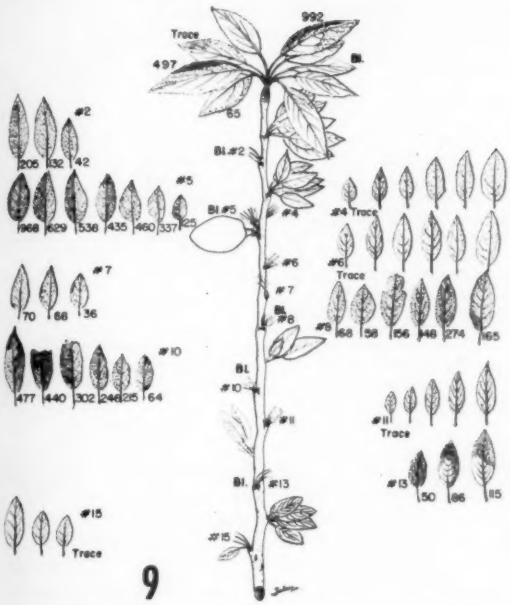

Fig. 9–10. 9) Distribution of disease symptoms and radioactivity (numerical values in counts/minute) in almond leaves blighted by C^{14}-labeled *Rhizopus*. Blighted leaf clusters (Bl.) are on the same side of the shoot as the inoculated fruit. Such leaves are highly radioactive whereas the leaves without symptoms have only trace amounts of radioactivity. The stippled areas designate relative density and distribution of radioactivity. **10)** Autoradiograph of an almond shoot harvested 4 hours after its fruit (arrow indicates where fruit was attached) was inoculated with C^{14}-2,3-fumaric acid. Note the unilateral distribution of the labeled acid in the leaves on the same side as the inoculated fruit.

Subsamples of the resulting radioactive mycelium were inoculated into almond hulls to determine whether the fumaric acid induced by infection was primarily of fungal or host origin. Control inoculations were made using radioactive mycelium killed by steam.

Inoculations were made in mid-August into fruits of Ne Plus Ultra on orchard trees. In order not to leach out the endogenous labeled material, the mycelium used as inoculum was not washed after it was taken from the medium. During transport to the orchard, the mycelium was kept moist. At the time of inoculation, the fruit had just begun to dehisce; as stated earlier, natural infection readily occurs at this stage of fruit development.

The amount of radioactivity in each leaf was determined by autoradiography and by assaying the activity of a representative disc of each leaf with a thin-end-window Geiger-Mueller tube. The dry weight of each leaf and its respective disc was determined. Then, the radioactivity in CPM of the leaf disc was multiplied by a factor obtained by dividing the total dry weight of the leaf by the dry weight of the leaf disc.

Hull rot and twig blight disease symptoms occurred after 7 days in 4 of the 6 fruits inoculated with the labeled mycelium. All controls (inoculated with steam-killed mycelium) were negative except 1 that became contaminated with naturally occurring *Rhizopus*.

Unilateral disease symptoms developed in the adjoining shoot and its leaves similar to natural infection. Moreover, leaves with symptoms were radioactive whereas those without symptoms had trace to 0 amounts of radioactivity (Fig. 9). In some leaf blades with unilateral necrosis, most of the radioactivity was restricted to the necrotic tissue (Fig. 11). In the control inoculations, radioactivity was detected only in the leaf cluster immediately adjacent to the inoculated fruit, and only in a trace amount.

In ethanol extracts from diseased leaves, all of which were radioactive, no fumaric acid was found. Results were similar in extracts of healthy leaves from control shoots. Malic and citric acids were present in large amounts in both the diseased and healthy control leaves. The malic acid from diseased leaves, however, was radioactive, whereas no radioactive malic acid was detected in the control and healthy leaves (Table 3). The significance of the radioactivity of malic acid is treated in the discussion.

These experiments confirmed the pathogenic role of *R. circinans* in the hull rot disease of almond. It showed that labeled carbon compounds produced by the fungus were translocated from the fruit into that part of the shoot and those leaves that develop symptoms. That fumaric acid is produced by *Rhizopus* growing on almond hulls has been shown in other experi-

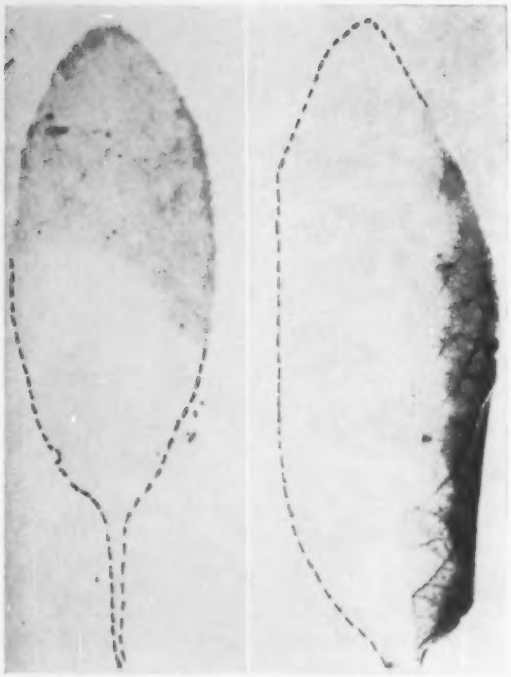

Fig. 11. Autoradiographs of leaves that developed necrotic zones as a result of inoculation of the fruit with C^{14}-labeled *Rhizopus* mycelium. Left, necrotic area on upper half of leaf had highest C^{14}-activity. Right, unilateral necrotic zone on right half of leaf shows a high accumulation of radioactivity.

ments. Although fumaric acid could not be detected in the shoot and leaves bearing symptoms, the data of the next experiments showed that it was translocated to these tissues and was converted to other metabolites prior to symptom development.

QUANTITATIVE DETERMINATION OF FUMARIC ACID. —*Naturally rotted hulls from orchard trees.*—Association of the toxin (fumaric acid) with the pathogen (*Rhizopus*) having been demonstrated, the problem remained to determine whether the fumaric acid in the diseased tissues was sufficient to be toxic. Since the fungus was found only in the almond fruit mesocarp, it was evident that toxin was produced in the fruit.

In August of 1959, hull rot and twig blight occurred in an almond orchard near Westley, Calif. The hulls (mesocarp) of these fruits were severely rotted, with *Rhizopus* spp. sporulating abundantly on their inner surfaces. Infected hulls adjacent to blighted shoots were harvested and analyzed for fumaric acid. Extractions also were made from apparently healthy hulls from the same trees. The hulls were air-dried for 7 days at 25–28°C and extracted with 70% ethanol.

Paper and gas-liquid chromatographic analyses revealed large amounts of fumaric acid in the infected hulls and only trace amounts in the controls (Table 4). These analyses do not take into account fumaric acid previously translocated to the stem and leaves.

TABLE 3. Distribution of radioactivity in various fractions of an extract of almond leaves after the nearest fruit was inoculated with C^{14}-labeled *Rhizopus*

Fraction	C.P.M.[a]	% of recovered activity
Inoculated with viable mycelium[b]		
Neutral and cation	94,905	89.6
Anion	11,000	10.4
Malic acid[c]	6,500	6.1
Other	4,500	4.3
Total recovered	105,905	2.4[e]
Inoculated with killed mycelium[d]		
Neutral and cation	3,330	
Anion	None	
Malic acid[c]	None	
Total recovered	3,330	0.075[e]

[a] Counts/minute.
[b] Leaf extraction made after symptoms had developed.
[c] No radioactivity in citric or tartaric acid.
[d] Leaf extraction made after a time comparable to the above extraction.
[e] Percentage of recovery of total activity applied.

Fumaric acid in leaves before symptoms develop. —On September 9, 1959, fruits on orchard trees of the almond variety Drake were inoculated with 3 ml of a 0.2 *M* solution of fumaric acid by the method described earlier. To this were added 2.5 μc of C^{14}-2,3-fumaric acid in 25 μl of water. Five days later, after uptake of the acid by the fruits, the leaves were analyzed for distribution of radioactivity and organic acid content.

As much as 6,090 μg of fumaric acid/g dry wt was found in the leaves of the treated shoots, whereas none was detected in the control (Table 4). Furthermore, the fumaric acid in the treated leaves was radioactive (Table 5). This is strong evidence that fumaric acid was actually translocated from the fruit into the leaves. Appreciable radioactivity also was detected in malic, citric, and tartaric acids (Table 5).

The original intent was to determine the percentage of the fumaric acid actually reaching leaves after application to the fruit. This was not possible, however, because of apparent conversion of the fumaric acid to other metabolites in the leaves. Nevertheless, the experiment did show that fumaric acid was rapidly metabolized after it reached the leaves.

Fumaric acid in leaves after symptoms develpp. —Fruit on orchard trees of the variety Drake was treated with both C^{14}-labeled and nonradioactive fumaric acid in the manner described above. The extractions, however, were made from leaves after symptoms developed. Inoculations were made on August 8, 1959, in the University orchard at Davis. One of the shoots bearing an inoculated fruit was harvested 4 hours after application of the isotope to determine whether labeled compounds (presumably fumaric acid) had already moved out of the fruit.

Within 4 hours, C^{14} was found in the leaves situated on the same side of the stem as the treated fruit (Fig. 10), showing a relatively rapid movement of C^{14}-compounds from the fruit into the adjoining shoot. Symptom development in the other treated shoots was evident after 2 days, and the shoots were harvested

TABLE 4. Concentration of fumaric acid in leaves and fruits of almond after various treatments

Treatment	Fumaric acid (μg/g dry wt)
Hulls rotted by *Rhizopus* sp.	225.8
in orchard trees	2,300.0
Control	Trace
Detached hulls rotted by *Rhizopus* sp. in laboratory	461.0
Control	Trace
Blighted leaves on orchard trees	Trace
Healthy leaves	Trace
Extraction from leaves on shoots after the nearest fruit was inoculated with fumaric acid but before leaf symptoms developed	6,090.0
Control for the above	Trace
Extraction from leaves on shoots after the nearest fruit was inoculated with fumaric acid but after leaf symptoms developed	Trace
Control for the above	Trace
Extraction from leaves after the nearest fruit was inoculated with C^{14}-labeled *Rhizopus* and leaf symptoms had developed	Trace

and air-dried after 7 days. In paper- and gas-chromatographic analysis of the blighted leaves and comparable leaves of the control, no fumaric acid was detected (Table 4). Radioactivity, however, was found in other metabolites (Table 6).

Although large amounts of fumaric acid had been translocated out of the fruit and into the shoot before symptoms developed, none was detected in the leaves at this later time. Radioactivity also was associated with citric, malic, and tartaric acids. This supports the assumption that either fumaric acid disappears as a result of its toxic action or its metabolic derivatives are toxic. The radioactivity in tartaric acid has a special significance, as noted in the discussion.

DISCUSSION AND CONCLUSIONS.—Analyses of leaves blighted by natural infection by *Rhizopus* and C^{14}-labeled *Rhizopus* or induced by fumaric acid consistently showed that fumaric acid disappeared after production of symptoms in the leaves. That fumaric acid actually reached the leaves has been shown by analysis of leaf extracts after fumaric acid had been applied to the fruit but before symptoms developed. Furthermore, radioactive carbon extracted from diseased leaves, but originally present in fumaric acid, was found in malic, citric, and tartaric acids. This shows that fumaric acid is converted to metabolic derivatives in the host. Likewise, where disease symptoms in leaves were induced by fruits infected by C^{14}-labeled *Rhizopus*, radioactivity in organic acids was recovered only in malic acid. The mechanism accounting for the disappearance of fumaric acid is not known but may hold the answer to its toxicity.

Kahlenberg and True (8), in 1896, reported toxic effects of fumaric acid on *Lupinus albus* L. Cooper and Edgar (5), in 1926, found that fumaric acid had a higher germicidal effect on bacteria than some phenols, and that it affected rates of enzyme action and precipitated protein. They concluded that its action is not a pH effect but rather is due to its stereochemical configuration when compared with the action of its cis isomer, maleic acid. This suggests that the toxicity found in the present experiments on almond hull rot may be due directly to the action of fumaric acid on the host protein.

When C^{14}-2,3-fumaric acid was translocated from the fruit to the adjoining shoot and leaves, the free acid disappeared and the C^{14} was found in malic, citric, and tartaric acids. Martin and Foster (11) reported the production of trans-L-epoxysuccinic acid by fungi and its biological conversion to meso-tartaric acid. They suggested malate as the precursor. In view of the data showing radioactivity in malic and tartaric acids, the formation of epoxysuccinic acid seems feasible. The latter compound is highly reactive and can readily denature protein by reacting with OH, NH_2, and SH groups. Although the biological formation of epoxysuccinic acid from malic acid is chemically feasible, the production of epoxysuccinic acid from fumaric acid is more probable, because of the difficulty involved in removing a hydride ion (a strong bond) from malic acid in the formation of the epoxide. Other studies (11) have shown that epoxysuccinic acid is converted to meso-tartaric acid, which in turn may be converted to oxalacetic acid (18). An equilibrium exists between oxalacetic and malic acids, which may account in part for the radioactivity in malic acid.

The stereoconfiguration of tartaric acid recovered in these experiments has not been established. This is important because epoxysuccinic acid is the precursor of meso-tartaric acid.

It is concluded that fumaric acid acts as a toxin in the hull rot disease of almond (as caused by *Rhizopus*) since it has been isolated from diseased host tissue, characterized, and produced symptoms of the disease when introduced into the host. Some evidence also is presented that in diseased tissues fumaric acid may be metabolized to other substances via a biochemical pathway that differs from the conventional citric acid cycle.

As similar diseases of almond, apricot, and peach caused by *Monilinia fructicola* and *M. laxa* have been observed by the authors, it would be of considerable

TABLE 5. C^{14} distribution in various compounds in an extract of almond leaves after C^{14}-2,3-fumaric acid had been applied to the nearest fruit, but before leaf symptoms had developed

Sample	C.P.M.[a]	% of total activity[b] recovered
Neutral and cation fraction	152,000	19.4
Anion fraction	633,144	80.6
Malic acid	107,457	13.7
Citric acid	109,989	14.0
Tartaric acid	43,995	5.6
Fumaric acid	368,520	46.9
Other	3,183	0.4

[a] Counts/minute.
[b] Of the total activity applied, 14.1% was recovered.

TABLE 6. Distribution of C^{14} in various compounds in an extract of almond leaves after C^{14}-2,3-fumaric acid had been applied to the nearest fruit, but after leaf symptoms had developed

Sample	C.P.M.[a]	% of total activity[b] recovered
Neutral and cation fraction	116,328	55.5
Anion fraction	93,240	44.5
Citric acid	18,400	8.8
Malic acid	15,200	7.3
Tartaric acid	2,600	1.2
Fumaric acid	0	0.0
Other	57,040	27.2

[a] Counts/minute.
[b] Of the total activity applied, 3.7% was recovered.

interest to determine if fumaric acid also is the cause of tissue necrosis in these diseases. Such studies are now in progress.

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GROWTH AND SPORULATION OF GUIGNARDIA BIDWELLII

Paul G. Caltrider

Formerly, Department of Plant Pathology, Bacteriology, and Entomology, West Virginia University, Morgantown; now, Research Assistant, Department of Plant Pathology, University of Illinois, Urbana.

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SUMMARY

Mycelial growth and production of conidia, spermatia, and pycnosclerotia in culture were markedly affected by various environmental and nutritional factors. The optimum temperatures were 25°C for growth and for production of pycnidia and conidia and 30°C for production of spermatia and pycnosclerotia. Continuous light greatly increased the abundance of pycnidia and conidia. Germination of conidia in a yeast-extract medium was greatest at 30°C at pH 4.5–5.0. Synthetic media which favored

growth generally favored production of pycnidia and conidia and were generally less favorable for spermatia and pycnosclerotia. The amount of growth on grape-decoction agar increased, but sporulation decreased, with maturity of the grapes used. Grape agar prepared from immature grapes favored sporulation. In the field, pycnidia, conidia, spermatia, and pycnosclerotia were produced under favorable conditions from June through August. Attempts to induce the formation of perithecia in pure culture were unsuccessful. Perithecia developed on a few grapes incubated in the greenhouse.

INTRODUCTION.—The black rot disease of grapes, caused by *Guignardia bidwellii* (Ell.) Viala & Ravaz, has been known in America since the early nineteenth century. Since that time, the epiphytology and symptomatology of the disease have been thoroughly studied (1, 2, 4, 5, 6, 7). Reddick (6), in 1911, made a comprehensive study of the control and life history of the fungus in nature. Although he grew the fungus in culture, he did not observe spermatia and ascospores. Germination of the conidia was reported to occur within 10 to 12 hours during favorable conditions.

The development of pycnidia, conidia, spermatia, pycnosclerotia, and ascospores have frequently been observed in nature, but the nutritional and environmental requirements necessary for their production have not been studied (3, 6, 7). Several investigators have associated these structures with a definite part of the growing season or a certain stage in the maturity of the grape (6, 7). The present study was made to determine the effect of various nutritional and environmental factors on the growth and sporulation of the fungus in pure culture and in nature.

MATERIALS AND METHODS.—The isolate of *G. bidwellii* used throughout this study was obtained from an infected grape. A medium containing 10 g glucose, 2 g yeast extract, and 20 g agar/liter was used for the study of sporulation. Nutritional work was done on a liquid basal medium composed of glucose, 10 g; asparagine monohydrate, 2 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Fe^{++}, 0.2 mg; Zn^{++}, 0.2 mg; Mn^{++}, 0.1 mg; thiamine hydrochloride, 100 μg; biotin, 5 μg; distilled water to make 1,000 ml. The medium was adjusted to pH 6.0 before autoclaving.

The grape-decoction agar medium was prepared by mincing 300 g of grapes in a Waring Blendor and adding 20 g of agar and enough distilled water to make 1 liter. The pH was adjusted to approximately 4.5 before autoclaving. The Concord variety of *Vitis labrusca* L. was used throughout the investigation. All cultural experiments were carried out in duplicate at 25°C unless otherwise stated.

RESULTS.—*Germination of conidia.*—Conidia harvested from a 10-day-old culture grown at 25°C in continuous light on the glucose-yeast-extract agar were placed in yeast-extract broth (2 g/liter). Germination was considered to have occurred with the production of a germ tube. The percentage of germination increased progressively from 15° to 30°C, but above 30°C it sharply decreased (Fig. 1). During the first 36 hours of incubation, the percentage of germination increased at all favorable temperatures.

The germination of conidia also was markedly influenced by the pH and available nutrients. In distilled water, the maximum germination in 36 hours was approximately 10%; on a medium containing yeast extract, germination was 50%. Germination on extracts from grapes increased with increased maturity of the grapes. The optimum pH for germination was between 4.5–5.0 in the yeast extract broth.

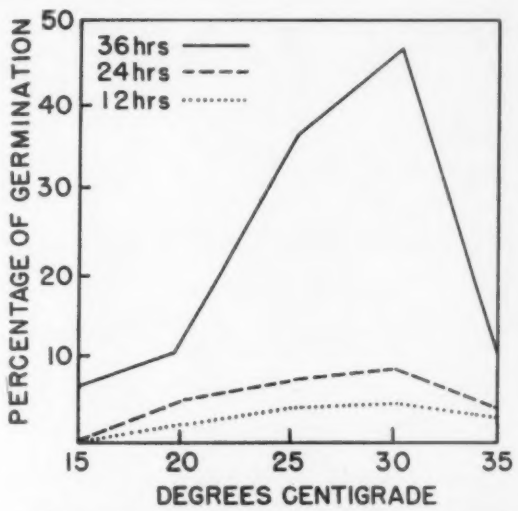

Fig. 1. Germination of conidia of *Guignardia bidwellii* on a yeast-extract agar at different temperatures for 12, 24, and 36 hours.

Effects of temperature and light on growth and sporulation.—Cultures were harvested from the liquid basal medium at approximately 3-day intervals and the dry weights determined. The minimum temperature for growth was below 15°C, the optimum approximately 25°C, and the maximum above 35°C (Fig. 2).

The abundance and type of fruiting structures varied with the temperature (Table 1). Some of the fruiting structures (true pycnidia) produced conidia; some (spermagonia) produced small spermatia-like cells; and others (pycnosclerotia) produced neither. Conidia were produced within the temperature range of 20–30°C, with the optimum near 25°C. Few fruiting structures formed below 20° or above 30°C. The optimum temperature for the production of spermatia and pycnosclerotia was approximately 30°C.

Continuous light from two 40-watt white fluorescent tubes at 15 in. greatly increased the production of conidia compared with alternate light and darkness. The oozing of conidia from the pycnidia was common under these conditions. Increased production of spermatia and pycnosclerotia was observed in cultures

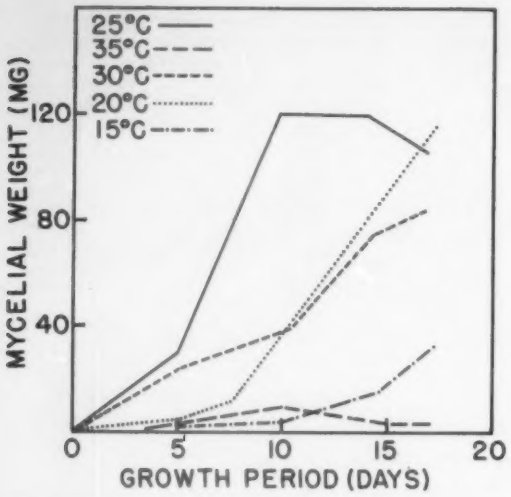

Fig. 2. Growth of *Guignardia bidwellii* at different temperatures on the liquid basal medium.

exposed to ultraviolet irradiation for 3 minutes. The effect of irradiation on production of conidia was less evident.

Effect of nutrition on growth and sporulation.—Previous workers have associated development of a certain spore stage of *G. bidwellii* with a specific time of the growing season or a certain stage of maturity of the grape (6, 8). Experiments were made to determine whether this phenomenon is due to nutritional or to environmental changes during the maturation of the grape. Grapes were collected at different stages of maturity at approximately 2-week intervals from July 1 to August 23. Cultures growing on grape-decoction agar prepared from the grapes collected at different stages of maturity gave distinct differences in the amount of growth. Growth increased progressively on the grape agar prepared from immature to the more mature grapes. These results indicate that the nutrients in mature grapes are more favorable for the growth of the fungus than are immature grapes.

The production of conidia, spermatia, and pycnosclerotia on the different grape-agars was generally less than on the glucose-yeast-extract agar. Media prepared from grapes collected late in the season generally were less favorable for the production of pycnidia and conidia than those prepared from grapes collected earlier. Spermatia and pycnosclerotia were generally more abundant on the grape agar prepared from grapes collected prior to August 1. These results closely paralleled the observations on sporulation in the field and the results obtained from inoculation.

The production of conidia was related to an abundance of mycelial growth on media varying in carbon and nitrogen sources. Those media which were less favorable for mycelial growth favored the production of spermatia and pycnosclerotia.

Production of ascospores in culture and on grape mummies.—Different isolates were paired, incubated at 25°C for approximately 2 weeks, then placed at 5° and 25°C. Some 2-week-old cultures also were spermatized with spermatia collected from a different isolate. These cultures were incubated at 25°C 3 days after spermatization, then placed in the same conditions as the paired isolates. Approximately 2 months later, the cultures placed at 5°C were moved to 25°C and 2 weeks later examined for the presence of ascospores. All efforts failed to produce the sexual stage in pure culture.

Grape mummies with abundant pycnidia were collected at approximately 2-week intervals throughout the growing season to determine whether ascospores are produced only on grapes infected at a certain stage of maturity. Approximately 25 grape mummies then were placed on soil at 5° and 25°C, and at the fluctuating temperatures of a greenhouse; and sprinkled periodically with water. After 5 months, the mummies kept at low temperatures were moved to the greenhouse; 2 weeks later, all the mummies were examined for ascospores of *G. bidwellii*. Ascospores were found only on 1 mummy, which had been kept at greenhouse temperatures. In another experiment, ascospores were found on the mummy of a grape which had been inoculated with a single isolate and placed under greenhouse conditions. Ascospores also were found frequently on grape mummies which had overwintered in nature.

Conidia were produced abundantly in the pycnosclerotia of the mummies after the long incubation period. This was also true of the mummies collected in nature.

Discussion.—A rather long warm, moist period is reported necessary for infection by *G. bidwellii* (6, 9). The slow rate of germination of the conidia would probably limit infection if extended wet periods did not occur. Since these prolonged wet periods are less common after midsummer, infection could be reduced even though conidia are present. Reddick (6) reported that asci were found in grape mummies throughout the summer. Therefore, the possibility of infection by the ascospores throughout the season cannot be eliminated.

The best temperature for production of pycnidia and conidia was approximately 25°C. Spermatia and pycnosclerotia were produced more abundantly at 30°C.

Table 1. The production of various fruiting structures of *Guignardia bidwellii* at different temperatures on a glucose-yeast-extract agar after 15-day incubation

°C	Pycnidia [a]	Spermatia [a]	Pycnosclerotia [a]
10	0	0	0
15	0	0	0
20	P	0	F
25	G	P	F
28	G	F	F
30	F	G	G
35	0	0	0

[a] 0 = none; G = good; F = fair; P = poor.

The latter structures have been reported to appear after midsummer (6). These studies indicate that both temperature and the nutrients present in the grape at different stages of maturity are important in determining the type of fruiting structure produced. Spermatia and pycnosclerotia were produced more abundantly on grape agar from the more mature grapes; pycnidia were favored by immature grapes. The significance of temperature and nutrients, in determining growth and production of various fruiting structures by the fungus in nature, is difficult to assess. The association of pycnosclerotia and spermatia with the latter part of the growing season and the reduction of infection of the grape with maturity tend to indicate the importance of these 2 factors. Other factors, such as morphological resistance, however, cannot be excluded.

Factors necessary for the production of ascospores were not determined. The production of a few ascospores under greenhouse conditions, however, indicates that low temperatures are not necessary for the development of the sexual stage. The large number of conidia produced in the pycnosclerotia after a dormant period could possibly play a more important role in primary infection than has been suspected. Reddick (6) reported that conidia may overwinter in old perithecia. In these studies, conidia were produced in the pycnosclerotia after a dormant period if placed in proper environmental conditions.

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REDUCTION IN WATER FLOW IN SOYBEAN STEMS BY A METABOLITE OF CEPHALOSPORIUM GREGATUM

Donald W. Chamberlain

Pathologist, Crops Research Division, ARS, U.S.D.A., Urbana, Illinois, in cooperation with the Illinois Agricultural Experiment Station and Department of Plant Pathology, University of Illinois, Urbana. Publication No. 350 of the U.S. Regional Soybean Laboratory, Urbana.

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SUMMARY

A study was undertaken to determine whether a metabolite of *Cephalosporium gregatum* could be responsible for reducing water flow in the vessels of soybean stems. An extract from diseased stems was forced through sections of healthy soybean stems, which then were tested for any change in the rate of water flow. Passage of the diseased-stem extract through the vessels reduced water flow to 10–34% of normal. The active agent was not removed by a Seitz filter and was still effective when the extract was diluted 1:5 and 1:10 with water; it was not destroyed or changed by autoclaving for 60 minutes at 15 psi.

In 1954, Chamberlain and McAlister (2) reported that brown stem rot, caused by *Cephalosporium gregatum* Allington & Chamberlain, reduced the rate of water flow in soybean stems. The question arose whether this reduction was caused by physical plugging of the vessels by fungus mycelium or by some metabolite produced by the fungus. This paper presents the detailed results of a series of studies designed to answer this question. The studies were briefly summarized in 1956 (1).

Materials and Methods.—Extract was obtained from soybean plants infected by *C. gregatum*. Infected, mature stems were soaked in water for 30 minutes and split longitudinally. The brown interior tissues (pith and vascular elements) were removed with a scalpel and homogenized in distilled water (10% w/v) in a Waring Blendor. A clear, liquid extract was obtained by straining the homogenate through absorbent cotton and centrifuging. For a control, an extract from healthy stems was made by the same method.

Water flow was measured in sections of healthy soybean stems cut with a sharp knife below the branch-bearing nodes. Each section, 7 cm long, included the second and third nodes. All sections were stored under water until used.

An apparatus, previously described in detail (2), forced water or the liquid extracts through the stem sections under 15 psi air pressure. A period of 30 seconds was allowed for any air present in the vessels to be expelled before water flow was measured. The amount (ml) of distilled water forced through the section in 4 minutes established the "normal" rate of water flow for each stem section. The extract then was forced through the stem section for 4 minutes; a second test was made with distilled water to determine any change in rate of flow. Unless otherwise specified, each extract was tested on 3–5 stem sections and each test was done 3 times.

In establishing the "normal" rate of water flow, repeated 4-minute runs with distilled water usually showed little variation in rate of flow (0–5%) from the initial run. Occasionally, differences up to 24% occurred. Consequently, as a precaution, differences in rate of flow not substantially greater than 24% were not considered significant.

PROPERTIES OF THE EXTRACT.—*Effect on water flow.*—The effects of extracts from diseased stems and from healthy stems were tested on 10 healthy-stem sections. The diseased-stem extract reduced water flow to 10–34% of the normal rate. Healthy-stem extract was relatively ineffective in reducing the flow rate (Table 1).

Stability to heat.—Samples of the extract were autoclaved 25 minutes at 15 psi pressure (120°C), cooled to approximately 25°C, and tested for effect on water flow in healthy-stem sections. The active principle was unchanged in its capacity to reduce water flow (Table 1). Further trials demonstrated that autoclaving the extract for 60 minutes induced no change in this respect.

Filterability.—The extract was filtered through a Seitz filter (sterilizing-type filter sheets) and tested for effect on water flow. The active principle was not removed by this type of filtration (Table 1).

Tolerance to dilution.—Seitz-filtered and autoclaved samples of the diseased-stem extract retained the capacity to reduce water flow when diluted 1:5 and 1:10 with distilled water. The 1:5 dilution appeared nearly as effective as the undiluted extract. The 1:10 dilution likewise reduced water flow but its effect was less pronounced than that of the 1:5 dilution (Table 1).

Production on agar medium.—*C. gregatum* was grown for 3 weeks on soybean-stem agar (1% w/v green soybean stems in distilled water homogenized in a Waring Blendor and strained through cotton, with agar added to make 2%). Three petri-dish cultures were homogenized in 150-ml distilled water, filtered through absorbent cotton, and Seitz-filtered. This filtrate reduced water flow in soybean stem sections to about the same extent as the 1:5 dilution of diseased-stem extract (Table 1). As a control, extracts made from sterile soybean-stem agar, on which no organism had grown, were tested for effect on water flow. The reduction in flow was comparable to that of the 1:10 dilution of diseased-stem extract (Table 1).

TABLE 1. Effect of extracts on rate of water flow in soybean stem sections

Type of extract	Avg.[a] "normal" rate of water flow (ml in 4 minutes)	% of "normal" rate of flow after treatment		
		Max.	Min.	Avg.
Centrifuged BSR[b]	49	34	10	18
Healthy stem	54	80	70	75
Autoclaved BSR	65	30	14	21
Seitz-filtered BSR	73	30	12	20
BSR diluted 1-5	39	42	18	27
BSR diluted 1-10	35	46	27	40
Cephalosporium gregatum agar	31	37	17	26
Sterile agar	32	66	26	44

[a] Average of 4 trials.
[b] BSR refers to extract from brown stem rot-infected stems.

Effect of repeated passages of the extract and permanence of the effect on water flow.—The effect on the vessels of longer exposure to the extract was determined by forcing the extract through the stem sections for 3 consecutive 4-minute periods. The amount of extract forced through the stem section was measured after each period. The maximum reduction in flow was attained after the second period. Usually, no further reduction occurred after the third period. Repeated (4) passages of distilled water thereafter did not increase the rate of flow. Stem sections through which the extract had passed were held under water in the refrigerator for 7 days and retested with distilled water. The rate of flow was not changed by this treatment.

Toxicity test.—In a test to determine toxic properties, 6 soybean plants 2 weeks old were cut off below the cotyledonary node. The bases of the stems were immersed in the diseased-stem extract for 5 days. Six control plants were immersed in distilled water for a similar period. No wilting was observed in any of the plants, but the plants held in the extract were browned at the severed end. When these stems were split longitudinally, internal browning was evident 6–8 cm above the base of the stem. None of the plants held in distilled water was discolored.

CONCLUSIONS.—These investigations indicate that the reduction in water flow in soybean stems affected by brown stem rot is caused by a metabolite of *C. gregatum*, rather than by physical plugging of the vessels by fungus mycelium. The effect of the metabolite on the vessels appears irreversible, since flushing the treated stem segments or soaking them in distilled water did not restore the original "normal" rate of water flow. The identity of the active principle in the extract and the nature of its effect on the vessels re-

main to be investigated. Since the extract did not induce wilting in live soybean plants, the principle apparently is not a toxin in the generally accepted sense of the term.

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THE PRESIDENT'S COLUMN

NO APOLOGIES

Too often these days, one hears from our members such statements as "plant pathology is only an applied science," or "our job is to serve other agricultural departments," or "after all, plant pathologists cannot expect equal recognition with other biological sciences since plant pathology is not a science in its own right."

I protest this apologetic attitude on the part of our own membership. If we take this attitude, can we object if those in other sciences comment in a similar vein? It seems to me this is the time for plant pathologists to proclaim vigorously their rightful place in science along with scientists in other fields of experimental biology, in chemistry, physics, and in science in general.

Earlier in the development of the science of plant pathology, the plant pathologist was the general practitioner or trouble-shooter for problems in crop agriculture. His unusually broad training in agricultural sciences provided him with an exceptional capacity to diagnose problems and to lead the way to their solution. He was able to mobilize knowledge in many fields of science, to integrate them, and to bring to bear such experience in meeting threatening disease emergencies in crop production. Where information was lacking, the plant pathologist had the versatility and scientific training to undertake research himself, in a number of different fields, in order to obtain the facts he needed.

Today, as earlier, the Ph.D. in plant pathology still has as broad, or broader, training as any in the agricultural sciences. He continues in demand, therefore, for the same capacities for diagnosis and recommended therapy, prevention, and eradication of diseases as before. But more and more, he is having to devote himself to extended research activities in order to build a greater reservoir of basic information. He requires more facts and principles on which to draw if he is to improve on the present degree of control of plant diseases.

One way in which the plant pathologist has proved himself many times a scientist in the highest sense has been through his contributions in such fields as genetics (inheritance of disease resistance in plants, inheritance of virulence, pathogenesis, and variation in plant pathogens), mycology and bacteriology (life cycles, physiology, taxonomy, antibiotics, and soil microbiology), virology (nature of viruses, transmission, serology, electron microscopy, invasion, and multiplication in the host), biochemistry (nature of fungicidal action and relation of molecular structure to biological activity), and ecology (influence of environmental factors on pathogenesis and survival of plant pathogens.) There are other areas all of you could add to this list, but these serve to bring to mind the many contributions plant pathologists have made to science, contributions which have been as profound, as fundamental, and as "pure" science as have been reported in these fields.

A second way in which the plant pathologist has proved his worth as a scientist is in the area of host-parasite relationships. This is a science in itself and is uniquely the plant pathologist's own field of science. The sum total of all basic research in mycology, physiology, and plant anatomy does not answer the pathologist's question of how a fungus establishes itself in its host. The action, reaction, and interaction of host and pathogen is an increasingly important science and belongs wholly and indisputedly to the plant pathologist. He can go as deeply and as "purely" into this science field of his as he wishes; and is doing so, with remarkable successes.

Past President, William C. Snyder

Above we are pleased to present a guest column by our immediate Past President. Plant pathologists have done well in the past and we hope that they will continue to do well in the future. As pointed out, however, it is becoming increasingly important for the plant pathologists to obtain more and more background in the basic sciences. By so doing, the ambitious plant pathologist will advance both his profession and himself.

With this Column, we terminate our tenure in the pilot's seat of the Society. The Column has been somewhat of an experiment and we trust an experiment not without some merit. We have endeavored to keep you informed on various questions which we believe are of general Society interest. Some of these will undoubtedly have come before the Council and the members at large at the Biloxi meeting and we hope will be resolved by now. We have appreciated the fine support by the officers and the members and we conclude with best wishes to you all.

S. E. A. McC.

NEWS

Editor: K. W. KREITLOW, Plant Industry Station, Beltsville, Md.

The 8th International Congress of Microbiology will be held in Montreal, Canada, August 19-25, 1962. The Secretary General of the Congress is Dr. N. E. Gibbons, c/o Division of Applied Biology, National Research Council, Ottawa 2, Ontario, Canada.

Plant pathologist Thomas Sproston, University of Vermont, Burlington, participated in the organizational meeting of a new group interested in the chemistry of plant phenolics. Dr. Sproston attended the meeting at Colorado State University as a recipient of a National Science Foundation grant.

The Department of Plant Pathology of Rutgers University celebrates its 50th Anniversary this year. On October 2, approximately 65 past and present students and staff members marked the occasion with a program at the University. Dr. B. H. Davis, Chairman of the Department gave a brief summary of the present status and future plans. Plant pathologist, Glenn Pound, University of Wisconsin, presented a paper on "Fifty Years of Plant Pathology." Speaker at the evening banquet was Henry Clay Lint, recipient of the first Ph.D. in plant pathology from Rutgers University. Dr. Lint described his graduate student days prior to 1916, and his work in many foreign countries as a U. S. Agricultural Attache.

Plant pathologist A. F. Ross, Cornell University, returned September 15 from a sabbatical leave to the Netherlands. In addition to continuing his research on plant viruses at the State Agricultural University at Wageningen, Dr. Ross attended the 13th International Symposium on Crop Protection held at Ghent, Belgium, and visited plant virology laboratories in England, Scotland, West Germany, Switzerland, Belgium, France, and Denmark.

Visiting pathologist Klaus Domsch, Biologische Bundesanstalt, Kiel-Kitzeburg, Germany, is spending six months in the Department of Plant Pathology, University of California, Riverside, cooperating in research on the breakdown of fungicides in soil.

The National Institutes of Health awarded a grant of $70,744 for a 3 year period to O. H. Calvert and J. D. Baldridge of the Department of Field Crops, University of Missouri, Columbia. The grant provides for 6 plant growth chambers and sponsors studies in host-parasite relations of legume diseases.

Mycologist Richard P. Korf, Cornell University, will be on leave of absence from October 1, 1961 until February 1, 1962, to continue his study of the Discomycete flora of Asia. His travel will include Japan, Indonesia, and Thailand. He presented an invitational address on the Hemiphacidiaceae before the annual meeting of the Mycological Society of Japan in October. To support his research abroad, Dr. Korf received a National Science Foundation grant.

After 51 years of continuous service E. M. Stoddard retired from the staff of the Connecticut Agricultural Experiment Station on May 31, 1961. His many years of research on the control of fruit diseases with fungicides and his wide interest in plants and their ailments have made him excell in the diagnosis of disease in plants. Mr. Stoddard has served as President of the Northeastern Division of the American Phytopathological Society and as Councilor from that division to the parent society.

Phytopathologist Arden F. Sherf, Cornell University, is on sabbatical leave until February 1, 1962. He will be headquartered at the University of California, Davis, and will study vegetable disease problems, control measures, and extension methods in California, Oregon, and Arizona.

Plant pathologist Charles E. Logsdon, ARS specialist in subarctic plant diseases is now on duty at the Agricultural College of Norway, Vollebekk, where he will attempt to define common circumpolar disease problems. Dr. Logsdon will return to Alaska in October, 1962 where he has been stationed as a joint employee of the University for eight years.

In the September issue of the Camellian, the editors made the following note about Dr. A. G. Plakidas, Professor Emeritus of Louisiana State University concerning his article entitled, Present Status of Control of Camellia Petal Blight: "We have never published any article or material before that we took any more pride in than we do the following article by Dr. A. G. Plakidas of Louisiana State University. It is the finest and most complete article on the subject that we have ever been privileged to read. We are deeply grateful to this fine man who is so generally and favorably known throughout the agricultural and horticultural world. We know of no one so well qualified who has given so generously of his time and talents throughout the years. There is little wonder he is held in such high esteem throughout the camellia world—both in America and abroad."

Virus diseases will be investigated at South Dakota State College, Brookings, by G. B. Orlob. Dr. Orlob did his under-graduate work at the University of Bonn, Germany, and obtained his Ph.D. degree at the University of Wisconsin. Prior to joining the South Dakota staff, Dr. Orlob worked for the National Research Council in Canada.

Plant pathologist A. J. Riker, University of Wisconsin, Madison, recently presented the results of his 1960 world observation on tree diseases at a meeting held in Vienna, Austria.

Nematologist Donald P. Taylor, Assistant Professor, Department of Plant Pathology and Botany, University of Minnesota will join the staff of the Department of Plant Pathology, University of Illinois, Urbana, as Assistant Professor of Nematology. Dr. Taylor will conduct research, direct graduate students and teach a course in nematology.

Phytopathologist Samuel F. Jenkins, Jr., joined the staff of the Plant Pathology Department, Georgia Coastal Plain Experiment Station, Tifton, on September 20, 1961. Dr. Jenkins, a recent graduate of North Carolina State College, will work on control and related phases of black shank and other diseases of tobacco.

Pathologist G. W. Keitt, Emeritus Chairman and Professor of Plant Pathology, University of Wisconsin, for 25 years, and Mrs. Keitt have moved from Madison to 55 Fayerweather, Cambridge 38, Massachusetts, where they will remain for one year before settling in South Carolina.

Deaths—Joseph H. Bolick, 32, died of a coronary attack on August 17. Mr. Bolick worked as Assistant Plant Pathologist for the U. S. Department of Agriculture in Gainesville, Florida since 1958, specializing on diseases of floricultural crops and other ornamentals. He was well on his way towards the Ph.D. degree in graduate work in plant pathology at the time of his death.

Miss Helen I. Johann, 81, died September 24 at Madison, Wisconsin. She was employed by the U. S. Department of Agriculture from 1919 to 1944. During this time she was stationed at Madison where she cooperated with the University of Wisconsin in studies on corn, barley and wheat diseases, particularly those caused by the Fusarium-Gibberella group of fungi.

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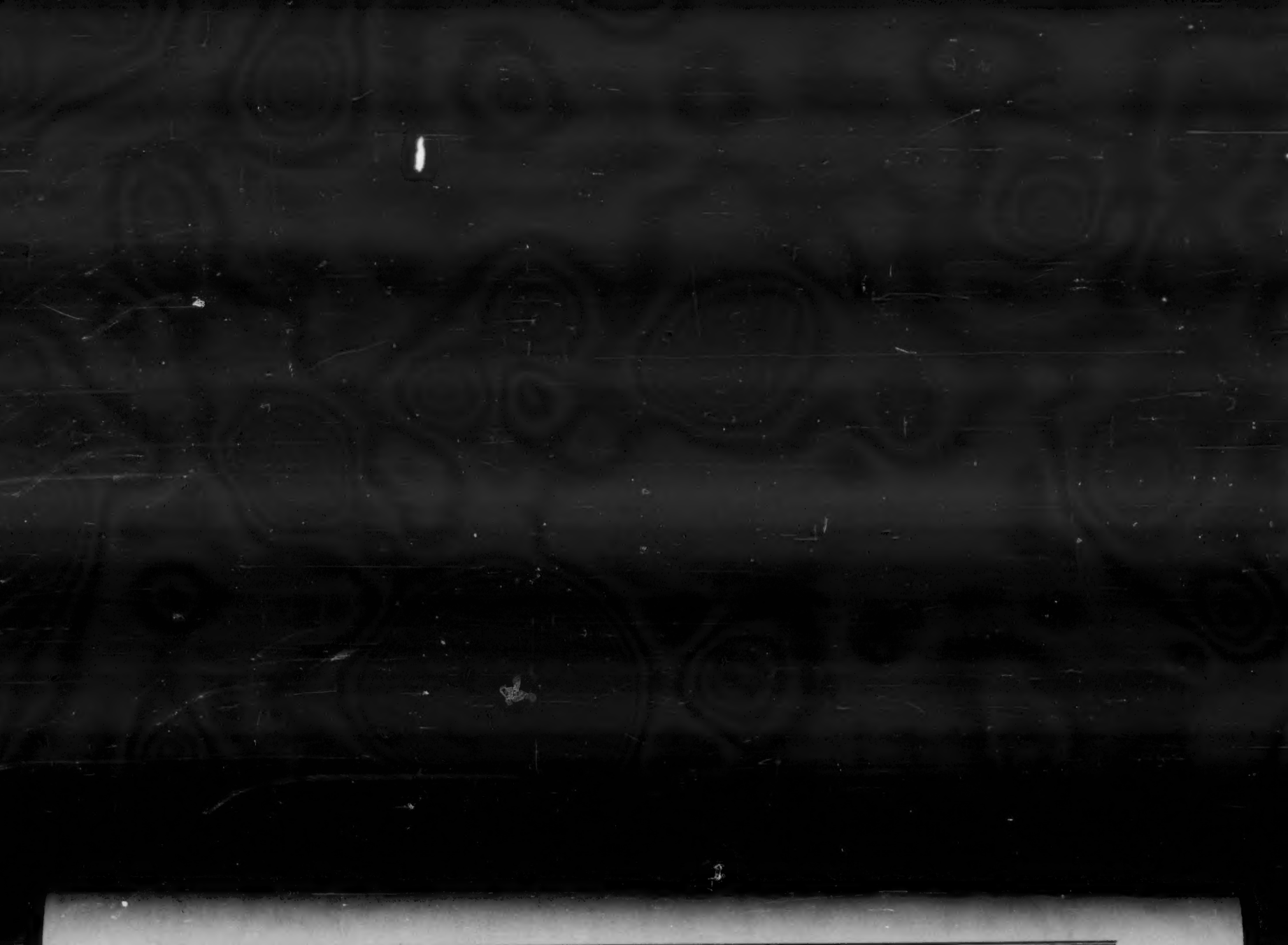
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